이학박사 학위논문

Role of PR3-homologous bacterial proteases in the formation of C-ANCA as a diagnostic marker for granulomatosis with polyangiitis

다발혈관염 육아종증 마커인 C-ANCA 형성에 관여하는 PR3 상동 세균 단백 분해 효소의 역할

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김 용 철

Abstract

Role of PR3-homologous bacterial proteases in the formation of C-ANCA as a diagnostic marker for granulomatosis with polyangiitis

Yong Chul Kim

Department of Immunology and Molecular microbiology in Dentistry

The Graduate School, Seoul National University

(Directed by Professor **Yongnim Choi**, D.D.S., Ph.D.)

Objectives

Molecular mimicry between microbial antigens and autoantigens has a role in many autoimmune diseases. Granulomatosis with polyangiitis (GPA) is a complex autoimmune syndrome that affects small- and medium-sized blood vessels in many tissues such as those in the upper respiratory tract, lung, and kidney. Anti-neutrophil cytoplasmic autoantibody (ANCA) antigens, especially those directed against proteinase 3 (PR3-ANCA) or myeloperoxidase (MPO-ANCA), are the hallmark of the GPA and are involved in the pathogenesis of vasculitis. In this study, the potential role of bacterial

proteases in the production of PR3-ANCA was investigated. Following a homology search of a bacterial protein database, two bacterial proteases homologous to PR3 were selected: the S1A family peptidase of *Myxococcus xanthus* (Mx) and the trypsin-like serine protease of *Saccharomonospora viridis* (Sv), which had 34%/44% and 30%/45% identities/similarities, respectively, with human PR3 and 33%/41% and 32%/43% identities/similarities, respectively, with mouse PR3. To elucidate the pathogenesis of GPA, two questions were addressed. First, although ANCA-mediated pathogenesis of vasculitis is well established, how is ANCA produced? Second, what is the pathogenesis of granuloma formation?

Materials and Methods

For this study, recombinant types of human PR3 (rhPR3), mouse pr3 (rmPR3), Sv protease (rSvPr), and Mx protease (rMxPr) were expressed in *Escherichia coli* and purified. NZBWF1 and C57BL/6 mice were intranasally mono-infected with either Sv or Mx bacteria or co-infected together with *Staphylococcus aureus* (Sa). In addition, mice were subcutaneously immunized with either rSvPr or Sv bacteria. In other experiments, mice were challenged once with Sa (nasal), Sv (nasal), or LPS (intraperitoneal) two weeks after immunization with rSvPr. Levels of IgG antibodies to purified human PR3 (phPR3), rhPR3, rmPR3, rMxPr, and rSvPr in sera were measured by using enzyme-linked immunosorbent assay (ELISA). For histological examination, sections of paraffin-embedded kidney and lung tissues were stained by using H&E or Verhoeff-Van Gieson (VVG) stains. Levels of antibodies to pHPR3, rHPR3, rmPR3, rMxPr, and rSvPr in GPA patient sera were measured by ELISA.

Results

Although all infected mice produced antibodies to bacterial lysates, only a small percentage of mice developed antibodies to bacterial proteases. Autoantibodies to rmPR3 were detected in 22.7% of NZBWF1 and 9% of C57BL/6 mice infected with Mx and in 33.3% of NZBWF1 and 13% of C57BL/6 mice infected with Sv. Differences in the percentage of autoantibody production associated with mouse strain or bacteria were not statistically significant. Mice infected with bacteria exhibited perivascular inflammation in the lung, but no granulomas or vasculitis were observed, even in mice that developed a high level of anti-mPR3 autoantibodies. Co-infection of Sa with Mx or Sv did not result in a significant change in the percentage of autoantibody production. Therefore, co-infection with Sa did not enhance the production of ANCA in mice. Interestingly, one C57BL6 mouse co-infected with Sv and Sa developed a large necrotic granulomatous lung lesion. In contrast to the results of bacterial infection, immunization with rSvPr resulted in the production of autoantibodies to rmPR3 in 75% of NZBWF1 mice and 100% of C57BL/6 mice. Mice immunized with rSvPr developed multiple inflammatory infiltrating sites in lung parenchyma and exhibited perivascular inflammation and vasculitis. The mice challenged once with Sa, Sv, or LPS following immunization with rSvPr developed vasculitis and small granulomatous lesions in lung parenchyma. Interestingly, antibodies to rSvPr were detected in 23% of PR3-ANCA GPA patients, but none were observed in control subjects. However, the percentage (63%) of control subjects with antibodies to Sv lysates in sera was higher than the percentage (38%) in PR3-ANCA GPA patients.

Conclusion

In mice, infection with bacteria containing PR3-homologous proteases or immunization with bacterial PR3 homologous proteases can induce PR3-ANCA production via molecular mimicry. Although elevated ANCA production did not induce granuloma development, ANCA alone could produce inflammation in the lung, which presented as inflammatory cell infiltration in the perivascular areas and vasculitis involving small- to medium-sized blood vessels.

Keywords: Granulomatosis with polyangiitis (GPA), Autoantibodies to neutrophil cytoplasmic antigens, Molecular mimicry, Proteinase 3

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1. Introduction

1.1. Activation of B cells and production of antibodies

When a microbe or antigen enters the human immune system, the human immune system activates naïve B lymphocytes. Those activated B cells differentiate into antibody-producing plasma cells and memory cells. Some antibody-producing B cells migrate to bone marrow where they can live for several years, during which time they continue to produce antibodies, even if the initial antigen has been eliminated. Consequently, if the same antigen again enters the body, the already circulating antibodies can provide instant protection against the antigen. Simultaneously, extant memory cells are activated by the antigen, and the resulting secondary response provides a high level of protection [1–2].

For an antibody response to a protein antigen, participation of both T and B cells is needed. Protein antigens that require the participation of T cells (CD4 T lymphocytes, T_{FH} cells) for immune response are referred to as T-dependent. However, an antibody response to a non-protein antigen, such as a polysaccharide or lipid, does not require participation of antigen-specific helper T cells; such antigens are referred to as T-independent [3–5].

Protein antigens require recognition of the antigen by the T_{FH} cells, which provide a co-stimulatory signal to antigen-specific B cells in secondary lymphoid tissues. The activated B cells undergo clonal expansion, isotype switching, affinity maturation, and differentiation into memory cells. At an early stage, the proliferation, initial antibody secretion, and isotype switching of B cells occurs in primary lymphoid follicles; subsequently, further proliferation along with affinity maturation and production of memory B cells occurs in the germinal center of lymphoid follicles [6–8].

1.2. B-cell tolerance

Development of B cells occurs in the bone marrow and development mechanism can be divided into various stages based on the makers of cell surface and intracellular markers [9–12].

1.2.1. Central tolerance of naïve B cells

Naïve B-cell generation in human bone marrow occurs via a strictly controlled developmental process. In bone marrow, the recognition of antigens by immature B cells is critical to the development of an immunological tolerance to self by negative selection. Central B-cell tolerance refers to a process that negatively selects newly generated immature B cells that will react to a self-antigen in the bone marrow environment. This step is considered a checkpoint in B-cell development, and the results of such checks are basic to the production of a naïve B-cell repertoire that contains foreign reactive antibodies, but that is largely empty of self-reactive specificities. This process produces B cells that do not react with self-antigens, but that may recognize antigens from pathogens (i.e., non-self).

Immature B cells express only IgM molecules on the surface and undergo negative selection by recognizing self-molecules present in the bone marrow. Self-antigen recognition results in a loss of cells from the B-cell repertoire and is referred to as clonal deletion. Negative selection of autoreactive B cells includes at least three distinct molecular mechanisms: deletion, anergy, and receptor editing. Several researchers have reported that the deletion of isolated immature B cells cultured with anti-BCR antibodies results in apoptosis. Immature B cells possessing a transgenic immunoglobulin receptor that binds soluble antigens undergo a short-lived anergic state.

Otherwise, self-reactive immature B cells can avoid deletion by inducing the expression of RAG-1 and RAG-2, which initiates a new VJ gene rearrangement resulting in a change to the autoreactive B-cell antigen receptor [13–20].

Osmond reported that wild-type mice produce about 2×10^7 immature B cells per day in their bone marrow. Among those cells, only 10% (about 2×10^6) can reach the periphery. Interestingly, among the immature B cells at the periphery about 25% have undergone receptor editing; thus, about 0.5×10^6 cells have undergone receptor editing [21–22].

1.2.2. Peripheral tolerance of naïve B cells

Transitional B cells are a type of immature B cell that can be translocated from bone marrow to spleen. All transitional B cells express CD93, whereas mature B cells do not express CD93. Moreover, all transitional B cells express a high level of IgM and a low level of IgD.

Based on their surface markers, transitional B cells can be subdivided into three types: T1, T2, and T3. In addition to expressing CD93, and in contrast to T1 cells, T2 and T3 cells express CD21 and CD23 [23–25]. Many researchers have reported that the anti-IgM antibody can trigger transitional B cells to undergo apoptosis [26–27]. Others have reported that only 10% of transitional B cells undergo maturation and become long-lived, mature B cells [28]. In peripheral tissues and lymph nodes, mature B cells are subjected to peripheral tolerance. If the mature B cells in peripheral tissues recognize self-antigens in the absence of specific T_{FH} cells, those B cells may undergo anergy or apoptosis. Moreover, because self-antigens alone cannot evoke innate immune responses, mature B cells will not receive cytokines or signals from T_{FH} cells. It has also

been reported that when B cells recognize self-antigens with a low affinity, the B cells would express various inhibitory receptors such as CD22, which results in a B-cell activation-threshold increase [29–31].

The B-cell activating factor (BAFF) and the BAFF receptors have an important role in the differentiation of transitional B cells into mature B cells. It has been reported that development of immature B cells of BAFF- and BAFF-receptor-deficient mice stops at the T1 stage in transitional B cells. Interestingly, when BAFF is overexpressed in mice, the mice developed a lupus-like disease [32–34].

1.2.3. Peripheral tolerance during affinity maturation of B cells

Affinity maturation is a process by which B cells produce antibodies with an increased antigen affinity within germinal centers via somatic hypermutation (SHM) of immunoglobulin (Ig) variable (V) region genes [35]. If an immune system is exposed repeatedly to the same antigen, the immune system will produce antibodies with increased antigen affinity by undergoing consecutive generation of plasma cells. It has been suggested that SHM and clonal selection have roles within the underlying mechanism. Hypermutation can occur in the complementarity-determining regions of Ig genes. The mutation rate of B cells within the lymphoid system is very high compared to that in cells outside the lymphoid system. Although, the exact molecular mechanism in SHM has not been described, activation-induced (cytidine) deaminase, an enzyme that can catalyze targeted deamination of deoxycytidine residues in deoxyribonucleic acid (DNA), has been reported to be part of that mechanism.

Clonal selection during affinity maturation involves follicular dendritic cells (FDCs) in the germinal center [36–38]. Mature B cells that receive self-antigens in

peripheral tissues in the absence of specific T_{FH} cells may be rendered functionally unresponsive or undergo apoptosis. Since self-antigens do not elicit innate immune responses, B cells will also not encounter any of the cytokines or other signals that may be induced during such responses. Thus, as observed in T cell studies, antigen recognition in the absence of a second stimulus results in tolerance. Many studies have reported that autoreactive B cells can be generated *de novo* during SHM in the germinal center. A post germinal center tolerance checkpoint has been proposed [39]. Within the peripheral tolerance autoreactive mature B cells in the germinal center, RAG can be re-expressed by exposure to interleukin-6 (IL-6). It has been reported that RAG expression can be induced by IL-6 treatment, but blocked by anti-IL-6 receptor antibodies in peripheral blood B cells of healthy individuals [40–42].

1.3. B cells in autoimmune disease

B cells can affect autoimmune diseases in various ways, such as secretion of autoantibodies, presentation of autoantigens, secretion of inflammatory cytokines, and modulation of antigen processing and presentation [43–48]. Cheema reported an elevated BAFF level in the serum of patients with an autoimmune disease. This suggests that B cells play an important role in the pathogenesis of autoimmune disease. Among the 185 patients in that study, which included patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), about 21% (38 patients) showed elevated BAFF levels in serum. The authors implied that overexpression of BAFF might result in a loss of B-cell tolerance [49].

B cells express CD20 (B-lymphocyte antigen CD20) on the surface, and rituximab is a chimeric monoclonal antibody against the CD20 protein. In 1997, the

United States Food and Drug Administration (USFDA) approved the use of rituximab to treat B-cell non-Hodgkin lymphoma [50]. Since that time, rituximab has been used to treat lymphomas, leukemias, transplant rejection, and autoimmune disorders (such as multiple sclerosis, SLE, and autoimmune anemias) because those ailments can eradicate B cells [51]. Moreover, rituximab has been shown to be effective in RA treatment [52].

1.4. Granulomatosis with polyangiitis

1.4.1. Definition and characteristics of granulomatosis with polyangiitis

Granulomatosis with polyangiitis (GPA) is a type of anti-neutrophil cytoplasmic autoantibody (ANCA)-associated systemic vasculitis that mainly affects small- and medium-sized blood vessels in many tissues. It is characterized by necrotizing inflammation of blood vessels and is associated with defects in respiratory system and kidneys. Clinically, there are two ANCA types: one is directed against proteinase 3 (PR3-ANCA) and the other against myeloperoxidase (MPO-ANCA). Generally, the PR3-ANCA is predominant in European GPA patients; however, the MPO-ANCA is predominant in Japanese patients [53–56].

1.4.2. Clinical features

Common upper-airway symptoms of GPA include nasal discharge and sinusitis. Pulmonary disorder, renal disease, and cutaneous manifestations are also common clinical features in GPA patients. Moreover, GPA patients often present with fatigue, fever, or joint pain (arthralgia) [57–59].

1.4.3. Pathological features

GPA is a complex autoimmune syndrome that is characterized by necrotizing granulomatous inflammation involving the upper and lower respiratory tract as well as focal glomerulonephritis and variable degrees of disseminated small-vessel vasculitis [60].

The histopathology of GPA includes the distinctive triad of necrotizing vasculitis, granulomatous inflammation, and glomerulonephritis. Granulomatous inflammations are reported to manifest during the early portion of the disease process, with vasculitis developing in later stages [61].

1.4.4. The pathogenesis of vasculitis: neutrophil-mediated vascular damage by ANCAs and cytokines

The pathogenesis model for vascular damage in GPA has been previously described. Initially, neutrophils and endothelial cells are activated by pro-inflammatory cytokines such as tumor necrosis factor (TNF) or interleukin-1 (IL-1) subsequent to infection or another inflammatory stimulus. The activated neutrophils express PR3 at the surface of the membrane. The expression of PR3 and MPO at the surface allows ANCA to bind the autoantigens, whereas the activated endothelial cells express E- and P-selectin. Moreover, the circulating ANCA binds to neutrophil membrane-associated Pr3 and to neutrophil fragment-crystalizable (Fc) receptors, thereby inducing neutrophil degranulation and generation of oxygen radicals. Briefly, after activation of neutrophils by ANCA, the activated neutrophils bind, adhere to, and kill endothelial cells via degranulation and oxygen radicals [62–64].

1.4.5. Epidemiology

In European populations, GPA is more common in men than in women, with a male-to-female ratio of 1.5:1. Although the onset of GPA may occur at any age, generally patients present within the age range of 35–55 years. Children are diagnosed with GPA rarely. GPA is a relatively rare disease with an undetermined incidence. The prevalence of GPA in the United States has been estimated to be 3 cases per 100,000 people, whereas the incidence and prevalence of GPA in the United Kingdom was estimated to be 10.2 cases and 250 cases per million people, respectively. In addition, GPA is reported to be more common in individuals of northern European descent [65–67].

1.4.6. Risk factors

1.4.6.1. Environmental exposures

Many possible provoking factors are associated with ANCA-associated vasculitis (AAV) such as silica, solvents, allergy, and vaccination. Some authors have reported that patients with chronic exposure to the ambient silica exhibit more disease activity and high serum ANCA titers with anti-MPO antibodies [68–69]. Moreover, patients taking hydralazine, propylthiouracil, or allopurinol show high anti-MPO antibody titers [70].

1.4.6.2. Genetic factors

Genetic factors associated with GPA have not been fully described. Among several candidate genes associated with GPA, the major histocompatibility complex, class II, DP beta 1 (HLA-DPB1) gene is the strongest genetic risk factor for developing GPA. The HLA-DPB1 gene belongs to the human leukocyte antigen (HLA) complex, which

generally assists the immune system to distinguish the body's own proteins from foreign antigens such as viruses and bacteria All HLA genes have several normal variations, allowing each person's immune system to react to a range of foreign antigens. A particular variant of the HLA-DPB1 gene called HLA-DPB1*0401 (rs9277554 region) has been detected more frequently in GPA patients, especially those with ANCAs, than in normal subjects. Moreover, a minor allele frequency (thymine) associated with GPA was reported to be lower in HLA-DPB1*0401 (rs9277554 region) subjects than in control subjects. Because the HLA-DPB1 gene is associated with the immune system, changes in that gene might be related to the inflammation and autoimmune responses that are characteristic of GPA. However, it is uncertain what role the HLA-DPB1*0401 gene variant has in the development of GPA [71-72].

1.4.6.3. Microbial factors

An association between GPA and bacterial infection was reported by Stegeman et al. [73]. In their study population, about 63% of GPA patients had chronic nasal carriage of *Staphylococcus aureus* (Sa), whereas about 25% of healthy controls and patients with other autoimmune diseases had chronic nasal Sa carriage [73]. Interestingly, increased levels of antibodies to several other bacteria including *Helicobacter pylori* were detected in GPA patients in a study by Lidar et al. [74–75]. In addition, treatment with co-trimoxazole (trimethoprim/sulfamethoxazole) is reported to reduce the incidence of relapse in GPA patients in remission [75]. In that study, 75% of the GPA patients (n = 16) in the co-trimoxazole group (960 mg, thrice a week) remained in remission at 18 months, whereas 55% of the placebo group (n = 15) remained in remission at 18 months.

1.4.7. Treatments

Generally, therapy for GPA patients can be divided into two stages. The first stage is undertaken to induce disease remission, and the second stage is intended to maintain disease remission. Typically, GPA patients are treated with immunosuppressive agents such as cyclophosphamide, mycophenilate mofetil, methotrexate, azathioprine, or glucocorticoids. Treatments are based on patient symptoms, disease severity, and organ involvement. Recently, the USFDA allowed the use of rituximab (Rituxan®) in the treatment of GPA and microscopic polyangiitis patients [76–79].

1.5. Molecularly mimicry

Classical molecular mimicry is a theoretical mechanism in which sequence homology between foreign molecules and self-peptides is adequate to result in cross-activation of autoreactive T or B cells by pathogen-derived peptides. The activation of B or T cells by self-homologous peptides suggests that activated B or T cell might cross-react with self-epitopes, thus resulting in antibody production and associated pathology in tissues or organs [80].

Molecular mimicry is reported to have a role in many autoimmune diseases as it can produce autoimmunity as the result of a homology between a pathogen and the host's protein. For example, it is reported that antibodies to the human lysosomal membrane glycoprotein 2 (hLAMP-2) are present on neutrophils and endothelial cells and can act as a specific indicator for pauci-immune crescentic glomerulonephritis (FNGN). Such neutrophils can be activated by anti-hLAMP-2 antibodies from rabbits. In addition, these antibodies produce FNGN in rats after immunization with recombinant

FimH. Interestingly, hLAMP-2 epitope ($P_{41–49}$) shows 100% homology with the bacterial adhesin FimH, an adhesion molecular of fimbriae of Gram-negative bacteria. It has been reported that the production of antibodies cross-reacting with hLAMP and resulting in FNGN was a consequence of immunization of rats with recombinant FimH [81].

1.6. Unanswered questions

There are three unanswered questions regarding the pathogenesis of GPA. First, although many researchers have reported on the pathogenesis of vasculitis, there are no reports that describe how ANCA is produced initially. Second, the causes of granuloma formation in lung and kidney in GPA patients have not been elucidated. Third, although ANCA is an important diagnostic and prognostic marker for GPA, there are no reports explaining the inconsistencies in the correlation between ANCA serum concentration and disease activity or relapse. Among these three questions, the first two are addressed in this study.

1.7. PR3-ANCA inhibits phagocytosis of bacteria by neutrophils

Results in a previous study showed that membrane-bound PR3 on neutrophils acts as a receptor for non-opsonic phagocytosis of bacteria. In that study, neutralization of membrane PR3 of human neutrophils with polyclonal ANCA reduced both bacterial binding and phagocytosis of bacteria such as *Streptococcus sanguinis*, *Porphylomonas gingivalis*, *Fusobacterium nucleatum*, and *Pseudomonas aeruginosa*. These results suggested the possibility that ANCA production may result from infection of some pathogens that possess a PR3-homologous protein [82].

1.8. Human PR3 is highly homologous to serine proteinases of many bacterial species

From a search of the microbial genome database for homologs to the PR3 protein sequence, many bacterial species with highly homologous proteinases were retrieved (Table 1). The S1A family peptidase of Mx had the highest homology with PR3 exhibiting a 34% identity and a 44% positivity with PR3. Mx is a Gram-negative bacterium commonly found in top soil. Among the species retrieved in the search, most were not human pathogens with the exception of Sv, *Vibrio cholerae*, and *V. vulnificus*. Sv is a Gram-negative bacterium frequently found in hot compost and hay. The spores of Sv can be readily dispersed in air, and prolonged exposure to those spores can lead to acute respiratory distress (farmer's lung disease), bagassosis, and humidifier fever [83–84]. The symptoms of those diseases are similar to the respiratory symptoms of GPA.

Table I. Bacterial proteases homologous to PR3

Bacteria	Protein	Identities (%)	Positives (%)	Expected protein size	E-value
Myxococcus xanthus DK1622	S1A family peptidase	34	44	30 kDa	$2x10^{-22}$
Streptomyces clavuligerus ATCC 27064	trypsin domain-containing protein	34	48	28 kda	$2x10^{-19}$
Pseudoalteromonas tunicata D2	secreted trypsin-like serine protease	32	48	61 kDa	$2x10^{-18}$
Saccharomonospora viridis DSM 43017	secreted trypsin-like serine protease	30	45	29 kDa	$5x10^{-17}$
Saccharopolyspora erythraea NRRL 2338	secreted trypsin-like serine protease	29	42	30 kDa	$9x10^{-16}$
Vivrio vulnificus	putative trypsin	29	47	60 kDa	$2x10^{-18}$
Vibrio cholerae RC385	putative trypsin	26	45	34 kDa	$2x10^{-14}$
Streptomyces avermitilis MA-4680	putative trypsin-like protease, secreted	27	44	29 kDa	$3x10^{-14}$

2. Materials and Methods

2.1. Serum samples from patients and control subjects

Serum sample were obtained from Department of Internal Medicine, Seoul National University Hospital, Korea. The disease control group contained 20 subjects (11 men and 9 women; mean age 41.3 years, range 20-72 years) and 20 GPA patients (11 men and 9 women; mean age 41.2 years, range 20-72 years). The study was performed according to ethical principles, after receiving approval from the
Institutional Review Board of Seoul National University Hospital (IRB number: 1010-033-335).

2.1. Bacterial strain and growth conditions

Mx DK 1622 was cultured at 32°C in CYE media. Sv DSM 43107 was grown at 45°C in nutrient broth. Sa ATCC 2713 was grown at 37°C in Luria-Bertani (LB) broth. All bacteria were cultured under aerobic conditions.

2.2. Production of recombinant proteins with a mutation in an active site

2.2.1. Primer design for the cloning of proteases

The primer sequences used are in Table 2. All underlined sequence at forward primers contained a kpn1 recognition site. The human PR3, mouse, PR3, and Mx protease were conducted active site mutations at first active site from histidine to alanine to prevent degradation of expressed proteins in *Escherichia coli* because of the proteolytic activity of proteases.

Table 2. Primer sequences for mutagenesis of recombinant proteins

Primer	Part 1 PCR	Part 2 PCR	Ligation PCR
Human Pr3	F-5' GGTACCATCGTGGGCGGGCACG R-5' AGCCGCGGC**CGT**CAGCACGAA	F-5' GCCGCG**CGT**TGCCTGCGGGACATACCC R-5' GCGCAGCGTGGAACGGAT	F-5' GGTACCATCGTGGGCGGGCACG R5'- GCGCAGCGTGGAACGGAT
Mouse pr3	F-5' GGTACCATTGTAGGTGGGCACGA R-5' AGCCGCGGC**CGT**CAGCACGAA	F-5' GCCGCC**CGT**TGCCTGCAGGACATCTCC R-5' TCAGGGCTCTGCGCCCCG	F-5' GGTACCATTGTAGGTGGGCACGA R-5' TCAGGGCTCTGCGCCCCG
Myxococcus Xanthus **S1A family peptidase**	F5'– GGATCCGGTCGATACGATCAGCCA R5'- AGCGGCAGC**CGT**GAGGACGATA	F5'- GCTGCC**CGT**TGTTTCTACGACGGTACGTCC R5'– CTATCTGACGGCGCTGTAGGTCC	F5'– GGATCCGGTCGATACGATCAGCCA R5'– CTATCTGACGGCGCTGTAGGTCC

Bold indicates mutation site

2.2.2. PCR conditions

Mutation PCR was performed for the recombinant human PR3, mouse pr3, and Mx protease except Sv protease. The first step PCR was performed using Takara Taq DNA polymerase (Japan) with the following conditions: 94°C for 5 min; 56 °C for 30s; 72 °C for 80 sec (repeated for 35 cycles). The primer sequences used are as Table 1. (First pair: Forward primer of flanking- Reverse of mutagenic primer, second pair: Reverse primer of flanking- Forward of mutagenic primer). The resulting two PCR fragments are gel-purified using Power Gel extraction Kit (Dyne Bio, Korea) for the second step of PCR. To combine the two PCR fragments prepared in the first step of PCR, a ligation PCR was performed using Pfu DNA polymerase (Stratagene, La Jolla, CA). The ligation PCR was performed with the following conditions: 98 °C for 2 min; 55 °C for 20 sec; 72°C for 1 min (Reapted for 30 cycles) using two PCR fragments (Result of first step of PCR) as templates. The products of liagated PCR fragment was gel-purified using Power Gel extraction Kit (Dyne Bio, Korea) for TA cloning. For the recombinant Sv protease the PCR was performed using Takara Taq DNA polymerase (Japan) with the following conditions: 94°C for 5 min; 56 °C for 30s; 72 °C for 80 sec (repeated for 35 cycles) using the following primers:

5’ –GGATCC GTGTTCACGAACATCCGGCGT and

5’ –TCACTCACCCGTTCTGCGTATCA. The products of PCR fragment was gel-purified using Power Gel extraction Kit (Dyne Bio, Korea) for TA cloning.

2.2.3. Cloning and expression of human PR3, mouse pr3, Mx protease, and Sv protease in *Escherichia coli*

The PCR products of human PR3, mouse pr3, Mx protease, and Sv protease were

cloned into the RBCTA cloning vector (Real Biotech Corporation, Tiawan). The plasmid DNA was isolated and digested with *Kpn1* and *Sal1* restriction enzyme. The fragments of proteainse genes were gel-purified using Power Gel extraction Kit (Dyne Bio, Korea). The purified genes were then ligated into the predigested pQE3- expression vector using T4 DNA ligase (Real Biotech Corporation, Tiawan) at 4 °C for 12 hours. *E. coli* SG13009 was transformed with the recombinant plasmids, and plated on LB agar plates containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin, and grown overnight. Next day, a single recombinant *E. coli* colony was grown in LB broth containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin until optical density (O.D) at 600 nm reaches 0.5-0.7, the expression of recombinant proteins were induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h. The proteins separated through a polyacryl amide gel were detected with coomassie blue staining and immunoblotting by using antibodies against histidine (Biolegend, CA, USA). After confirmation of the protein expression, 250 ml cultures of *E. coli* transformed with each genes were cultured until the optical density (O.D) at 600 nm reaches 0.5-0.7 After 1mM IPTG and 0.5 mM PMSF (phenylmethylsulfonyl fluoride, proteinase inhibitor) were added, the cultures were incubated for an additional 4 hour. The bacteria were harvested by centrifugation at 4000x g , 4°C for 20 min and the cell pellets were frozen overnight at -20 °C. The pelltes were suspended in 10 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing 100 μg/ml of lysozyme and then incubated at 4 °C for 15 min and subsequently sonicating them with an ultrasonic processor (10 sec bursts at output 20 W with a 10 sec cooling period between burst). After sonication, the lysates were centrifuged at 10,000 x g for 10 min, the supernatants containing the proteases were collected. The recombinant proteins (rmPr3, rhPr3, rMxPr and rSvPr) were purified under

native conditions using nickel-nitrotriacetic acid agarose (Ni-NTA agarose; QIAGEN, Valencia, CA, USA) according to the manufacturer's protocols. Briefly, the recombinant proteins bound to Ni-NTA agarose for 1 hour at 4°C were washed 5 times with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and then eluted with elution buffer(50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). All recombinant proteins were confirmed by electrophoresis through a SDS-PAGE gel and coomassie blue staining.

2.3. Endotoxin removal

For removal of endotoxin in the recombinant proteins, the Triton X-114 (Sigma, St. Louis, MO) two-phase extraction method was used. Those method is an efficient method to dissociate endotoxin from biomolecules including recombinant proteins. All recombinant proteins were extracted three times with Triton X-114 (at a final concentration of 1%). Endotoxin contamination in the rmPr3, rhPr3, rMxPr and rSvPr preparation was determined by Pierce Limulus amebocyte lysate (LAL) Chromogenic Endotoxin Quantitation Kit (Pierce Biotechnology, Rockford, USA) according to the manufacturer's protocols. In principle, bacterial endotoxin activates a proenzyme in the modified Lumulus Amebocyte Lysate (LAL). The activated proenzyme quickly catalyzes the splitting of p-Nitroaniline(pNA), which can converts a colorless substrate into a yellow color. The developed color intensity was measured at 405nm and calculated using *E. coli* endotoxin standard (011:B4) provided in the kit. The endotoxin level of recombinant proteins were below 0.2 EU/ml. The concentration of recombinant proteins was determined using a bicinchoninic acid (BCA) (Thermo scientific, Rockford, USA)

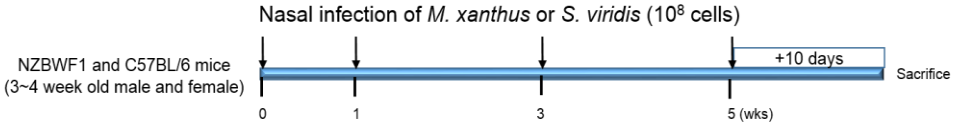
2.4. Mice

This study was conducted in accordance with the recommendations of the Guide from the Institute of Laboratory Animal Resources Seoul National University under the Laboratory Animals Act 9025 of the Republic of Korea. The experimental protocol and animal handling procedures were approved by the Seoul National University Animal Care and Use committee (SNU-120416-2).

Three-week-old NZBWF1 and C57BL/6 mice were purchased from SLC Inc (Japan) and Orient Bio (Orient Bio Inc., Seongnam, Korea), respectively and housed under specific pathogen-free conditions in the Laboratory Animal Facility at the School of Dentistry, Seoul National University. All groups in the experiments were mixed gender.

2.5. Bacterial infection

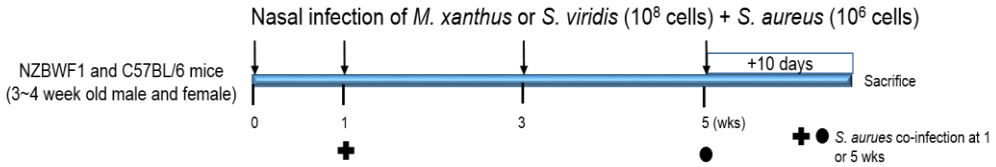
2.5.1. Mx or *Sv* infection



NZBWF1 mice were chosen because those strain mice have been used as a model for spontaneous autoimmune disease explaining not only the complex immunobiological responses and mechanisms but also the genetic foundation for the autoimmune diseases. C57BL/6 mice, on the other hand, were chosen as a normal control. Generally, the NZBWF1 mice show upraised serum antinuclear autoantibodies (ANA) level such as anti-dsDNA IgG (immunoglobulin), a majority of and immune complex-mediated glomerulonephritis (GN) at 5-6 months of age [66]. To exclude those features of NZBWF1

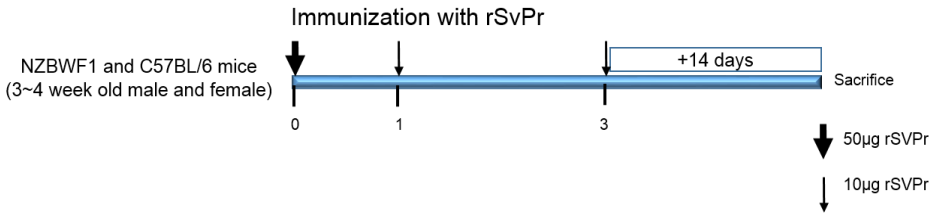
mice, three- to four-week old mice were randomly dived into three groups: sham, Mx, and Sv. The Mx or Sv group was intranasally infected by inoculation of 30 μl of10^8 cells of each live bacteria in phosphate-buffered saline (PBS) onto the nostrils at 0, 1, 3 and 5 weeks. The sham group was received quantities of PBS. All mice were euthanized 10 days after the last infection. Blood, lung and kidney samples were collected immediately after euthanasia.

2.5.2. Co-infection of Sa with Mx or Sv



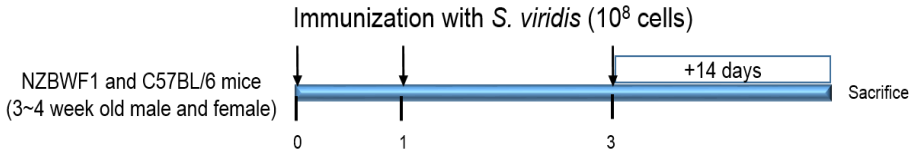
Three- to four- week old mice were randomly dived into three groups: sham (Sa), Mx+Sa, and Sv+Sa. The Mx+Sa or Sv+Sa group was intranasally infected by inoculation of 30 μl of 10^8 cells of *Mx* or *Sv* in PBS onto the nostrils at 0, 1, 3 and 5 weeks according to the sample protocol. The mice were co-infected with 10^6 cells of Sa together with Mx or Sv once at either 1 or 5 weeks. The sham (Sa) group was received PBS or Sa alone. All mice were euthanized 10 days after the last infection. Blood, lung and kidney samples were collected immediately after euthanasia

2.6. Immunization with recombinant Sv proteinase



Three- to four- week old mice were immunized subcutaneously with 50 μg of *Sv* protease emulsified with complete Freund's adjuvant (CFA) (Sigma, St. Louis, MO). One and three weeks later mice were boosted again with the 10 μg of Sv protease emulsified with CFA. The sham group was immunized equal quantities of vehicle emulsified with complete Freund's adjuvant. The immunized mice were euthanized 2 weeks after the last immunization. Blood, lung and kidney samples were collected immediately after euthanasia

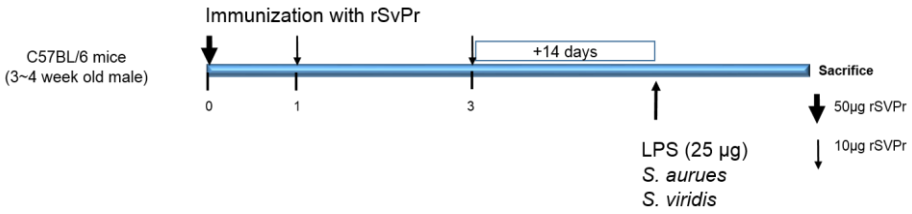
2.7. Immunization with Sv



Three- to four- week old mice were immunized subcutaneously with 10^8 cells of *Sv* emulsified with complete Freund's adjuvant (CFA, (Sigma, St. Louis, MO). One and three weeks later mice in each group were boosted again with the 10^8 cells of the same materials. The sham group was immunized equal quantities of vehicle emulsified with complete Freund's adjuvant according to the sample protocol. The immunized mice were euthanized 2 weeks after the last immunization. Blood, lung and kidney samples were

collected immediately after euthanasia

2.8. Sa (nasal), Sv (nasal), or LPS (Intraperitoneal) challenge to ANCA produced mice with rSvPr immunization



Three- to four- week old mice were immunized subcutaneously with 10^8 cells of *Sv* emulsified with complete Freund's adjuvant (CFA, (Sigma, St. Louis, MO). One and three weeks later mice in each group were boosted again with the 10^8 cells of the same materials. The sham group was immunized equal quantities of vehicle emulsified with complete Freund's adjuvant according to the sample protocol. ANCA- induced mice after challenged with Lipopolysaccharides (LPS), Sa, or Sv.

2.9. ELISA (Enzyme-linked immunosorbent assay)

Ninety-six-well plates (costar, New York, NY, U.S.A), were coated with purified human proteinase 3 (ML 374, Elastin Products Company, USA),rmPr3. rhPr3 and recombinant bacterial proteinase (*Sv* and *Mx*) at 2 μg/ml (diluted in a phosphate buffered saline) or with PBS alone or mouse IgG_1 as standards at 10, 8, 6, 4, 2, 1 ng/ml and incubated overnight at 4 ℃. Plates were washed 3 times with PBS-Tween 20 (0.1%) and blocked with 1% bovine serum albumin (BSA) diluted in PBS for 120 minutes at room temperature. Mice sera were diluted in 1:150 and 1:300 in blocking buffer, added in

duplicates, and incubated for 90 minutes at room temperature. Plates were washed as described above and incubated with peroxidase conjugated goat anti-mouse IgG (southernBiotech, Brimingham, U.S.A) (diluted 1:5,000 in blocking buffer) for 60 minutes at room temperature. After a final wash 5 times (PBS-Tween 20, 0.1%), bound antibodies were detected with tetramethylbenzidine substrate (Sigma, St. Louis, MO). The reaction was stopped by the addition of 2 N H_2SO_4, and Absorbance was measured at 450 nm in a Multiscan Ascent microplate reader. The OD_{450} values were calculated by the mouse IgG1 standards.

For the clinical samples, pPR3 (1ug/ml), Sv lysate (0.5 ug/ml), rSvPr (2ug/ml), rMxPr (2 ug/ml), rhPr3 (2ug/nl), Mx lysate (0.5 ug/ml), Sa lysate (1ug/ml), and without antigen in 0.05 M sodium carbonate buffer, pH 9.6, with 0.01% Triton X-100 is used for coating ninety-six-well plates (costar, New York, NY, U.S.A) and incubated overnight at 4 °C. Plates were washed three times with wash buffer (0.15 M NaCl containing 0.05 % Tween 20). Patients and controls sera diluted 1:1000 in incubation buffer (Tris 0.05 M, NaCl 0.15 M, Tween-20 0.05 %, bovine serum albumin 0.2 %) were added to duplicate wells and incubated for one hour at room temperature and then washed three times as above. Alkaline phosphatase-labelled goat anti-human IgG (Sigma, St. Louis, MO) diluted in 1:5000 in incubation buffer is added to each well and incubated at room temperature for one hour. The plates were washed three times as above and 100 ul paranitrophenyl phosphate substrate (1mg/ml) (Sigma, St. Louis, MO) in diethanolamine (Sigma, St. Louis, MO) 1 M, pH 9.8, is incubated for 30 min. at room temperature, after which OD 405 nm values are read in an ELISA reader.

2.10. Histology

The lung and kidney samples were fixed in 4% paraformaldehyde and embedded in the paraffin. Serial sections (4μm) of the paraffin-embedded kidney and lung tissues were stained by hematoxylin and eosin for histological examination.

For Verhoeff-Van Gieson (VVG) stain, Tissues sections were deparaffinized, rehydrated, and incubated with Verhoff's solution for 30 min and then rinse in tap water. The slides were differentiated in 2% ferric chloride solution for 90 sec, stopped differentiation with tap water and incubated with 5% sodium thiosulfate solution for 3 min. The slides were washed with tap water and counterstained with Van Gieson's solution. After dehydrate quickly through alcohol, clear with xylene and the mounted with mounting solution.

2.11. Statistics

All statistical analyses were performed with SPSS 18 software (SPSS Inc., Chicago, IN, USA). Data were analyzed using the Mann-Whitney U test to determine the differences between the sham and experimental groups in the animal study and the differences between the healthy subjects and the patients with GPA. Data were considered statically significant at a p-value of < 0.05.

3. Results

3.1. Production of recombinant proteases

Previously, it was reported that membrane-bound PR3 on neutrophils acts as a phagocytotic receptor for non-opsonic phagocytosis of bacteria. Following neutralization of PR3 with ANCA, binding and phagocytosis of bacteria is decreased [67]. Thus, it was speculated that ANCA could be produced following infection by bacteria containing a PR3 homologous protein. To investigate that possibility, a bacterial protein database was searched using the PR3 protein sequence as the query item. Many of the search-identified bacteria contained a PR3 homologous protease. Of the identified bacteria, two were chosen for further study: Mx and Sv. The S1A family peptidase of Mx had the greatest homology to PR3 with a 34% identity and 44% positivity with human PR3. Mx is a Gram-negative bacterium commonly found in top soil. The protease of Sv had 30 x% identity and 45 x% positivity with PR3. Sv is a Gram-negative bacterium frequently found in hot compost and hay. Spores of Sv can be readily dispersed in air, and the prolonged exposure to those spores can lead to acute respiratory distress (farmer's lung disease), bagassosis, and humidifier fever. Those symptoms are similar to the respiratory symptoms of GPA.

In order to use a mouse system during this study, the homologies among human PR3, mouse pr3, and bacterial protease were investigated. Mouse pr3 showed a 69%/81% identity/ positivity with human PR3. Moreover, mouse pr3 had 33%/41%, and 32%/43% identities/ positivity with the Mx and Sv proteases, respectively. Human PR3, mouse pr3, and the bacterial proteases were highly conserved at the active sites (Fig. 1 A).

When wild human PR3, mouse pr3, and Mx protease were expressed in *E. coli*, all proteases were toxic to *E. coli* because of their proteolytic activity. To reduce that proteolytic activity, the proteases' active sites were mutated from histidine to alanine (Fig. 1), with the exception of rSvPr, which was expressed without mutation. The expressed recombinant proteins were detected by using SDS-PAGE (Fig. 2). The molecular sizes of the recombinant proteins were approximately 25 kDa. Two contaminated SDS-PAGE gel bands at 55 kDa and 70kDa could not be removed by centrifugation through a membrane with 30 kDa pores. By using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis, the upper band (bold arrow) in Fig. 2 was identified as glutamine aminotransferase of *E. coli* and the lower band (thin arrow) was identified as GroEl of *E. coli*. One possible explanation for the presence of these bands is that the recombinant proteins may be bound to the contaminant proteins. A second possibility is related to GroEl's chaperon function. GroEL belongs to the molecular chaperonin family, is present in many bacteria, and is required for the proper protein folding during the expression of recombinant proteins.

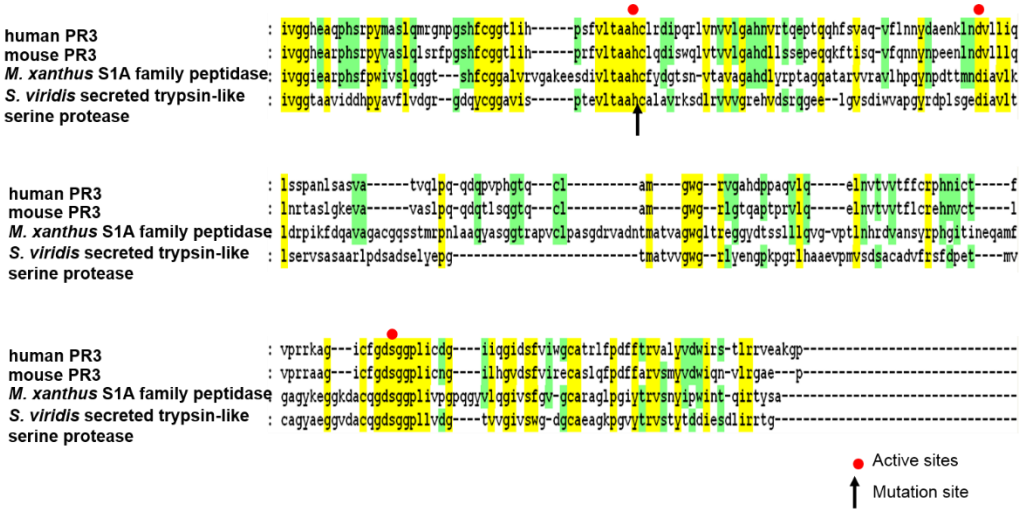


Figure 1. Homology of human PR3 with mouse R3 and bacterial proteases. The rmPr3, S1A family peptidase of Mx and the trypsin-like serine protease of Sv that have 69%/81%, 35%/45% and 31%/44% identities/similarity with PR3, respectively. The red circle indicates active sites of protease. The arrow indicates a mutation site introduced into the rhPr3, rmPr3, and rMxPr.

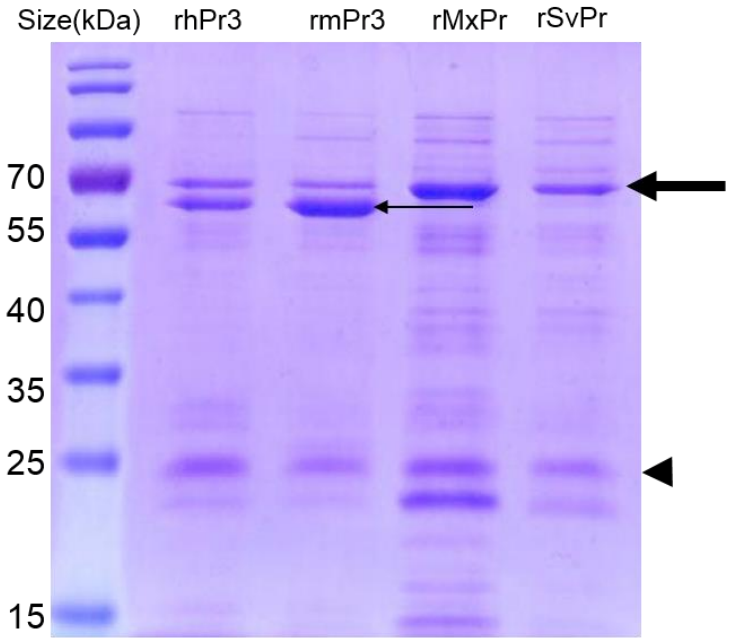


Figure 2. The recombinant HPR3, mPR3, MxPr, and SvPr are expressed in *E. coli*. After removal of LPS from the recombinant proteins, rhPr3, rmPr3, rMxPr, and rSvPr were separated through a 12% SDS-PAGE gel and stained with coomassie blue staining. The expected size of recombinant proteinases are around 25kDa (Arrowhead). (Bold arrow indicates glutamine aminotransferase of *E. coli*, thin arrow indicates chaperonin GroEL)

3.2. Induction of PR3-ANCA by nasal infection of Mx or Sv in the mice

3.2.1. Autoantibodies to rmPr3 was examined in the mice after repeated nasal infection of either Mx or Sv

In order to determine the infection schedule, mice were infected with Mx or Sv and collected mouse sera at various time points. Autoantibodies to rmPr3 were produced after second infection of Mx and forth infection of Sv (FIig.3). Therefore, I decided to observe antibodies after four times of infection with bacteria.

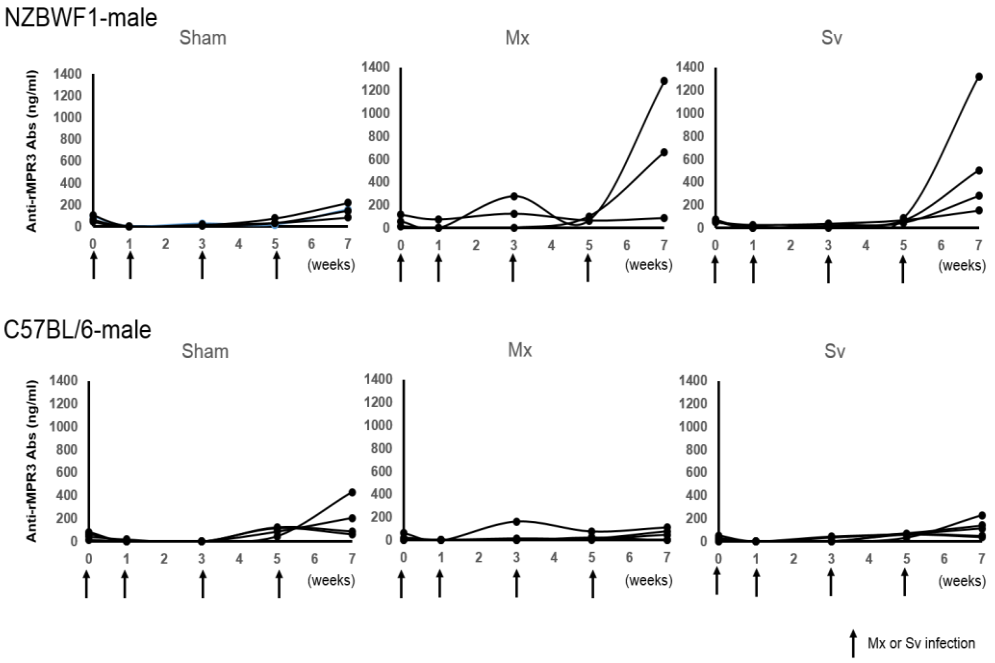


Figure 3. The level autoantibodies to mPR3 in sera of mice infected with Mx or Sv at various time point. The mice were infected with Mx or Sv at 0, 1, 3 and 5 weeks. Serum samples were collected at 0,1,3,5, and 7 weeks after first infection. Nasal infection with Mx or Sv induces production of autoantibodies to mPR3. The upper panel shows NZBWF1 strain and the lower panel shows C57BL/6 strain.

3.2.2. Nasal infection with Mx- or Sv-induced humoral response to bacteria

In order to verify the existence of humoral responses to bacterial infections, the presence of antibodies to bacterial lysates was examined. Nasal infection with Mx or Sv induced an antibody response to the Mx and Sv lysates. In both NZBWF1 and C57BL/6 mice strains, all mice infected with Mx produced antibodies to the Mx lysate (Fig. 4 A). Similarly, in both mouse strains all mice infected with Sv produced antibodies to the Sv lysate (Fig. 4 B). However, mice infected with Mx did not produce antibodies to the Sv lysate. Similarly, mice infected with Sv did not induce antibodies to the Mx lysate. Mice in the NZBWF1 strain showed higher levels of response to both bacteria than the responses in C57BL/6 strain mice.

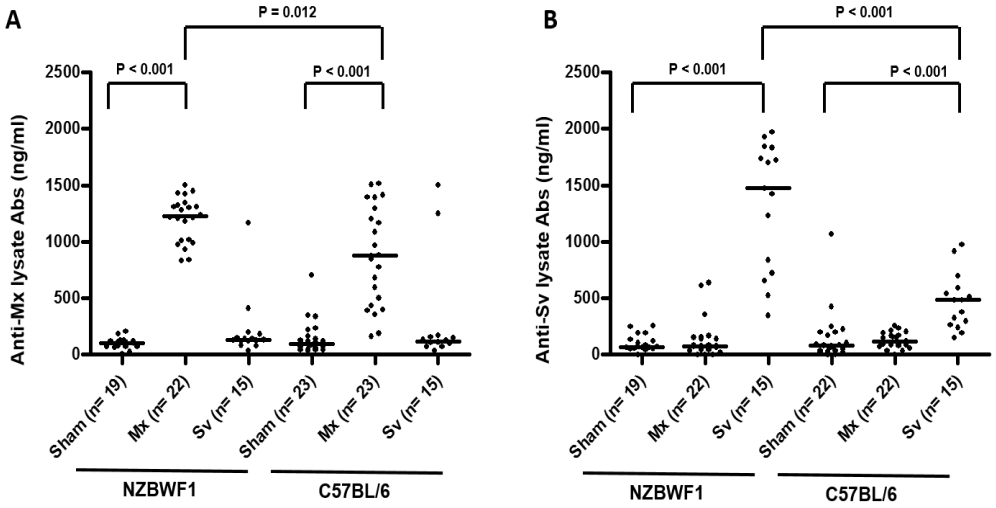


Figure 4. Nasal infection with Mx or Sv induced efficient humoral response to bacteria. The NZBWF1 and C57BL/6 mice were intranasally infected by inoculation of 30 µl of10^8 cells of Mx or Sv live bacteria in PBS onto the nostrils at 0, 1, 3 and 5 weeks. The sham group was received PBS. All mice were euthanized 10 days after the last infection. The levels of antibodies to Mx (A) and Sv (B) lysates in sera were determined by ELISA.

3.2.3. Nasal infection with Mx- or Sv-induced autoantibodies in only a few mice

In order to verify the existence of antibody responses to mouse PR3, human PR3, and the bacterial proteases, the presence of antibodies to rmPr3, rMxPr, rSvPR, rhPr3, and phPR3 was examined. The Mx-infected mice produced antibodies to rMxPr in 3 of 23 NZBWF1 mice and in one of 23 C57BL/6 mice (Fig. 5 A). Similarly, Sv-infected mice produced antibodies to rSvPr in three of 15 NZBWF1 mice and in one of 15 C57BL/6 mice (Fig. 5 B). Autoantibodies to rmPr3 were detected in 22.7% of NZBWF1 strain mice and in 9% of C57BL/6 strain mice infected with Mx. In mice Sv-infected mice, antibodies to rmPr3 were produced in 33.3% of NZBWF1 mice and 13% of the C57BL/6 mice (Fig. 5 C). Most anti-rmPr3 reactive sera from NZBWF1 mice, but not from C57BL/6 mice, reacted to rhPr3 and phPR3 (Fig. 5 D, E). Estimation of antibody responses to phPR3 was performed in the first bacterial set among three independent bacterial infected sample groups. The results indicate that few mice develop antibodies to the rmPr3 autoantigen or the homologous bacterial proteases despite all mice exhibiting an antibody response to the bacteria.

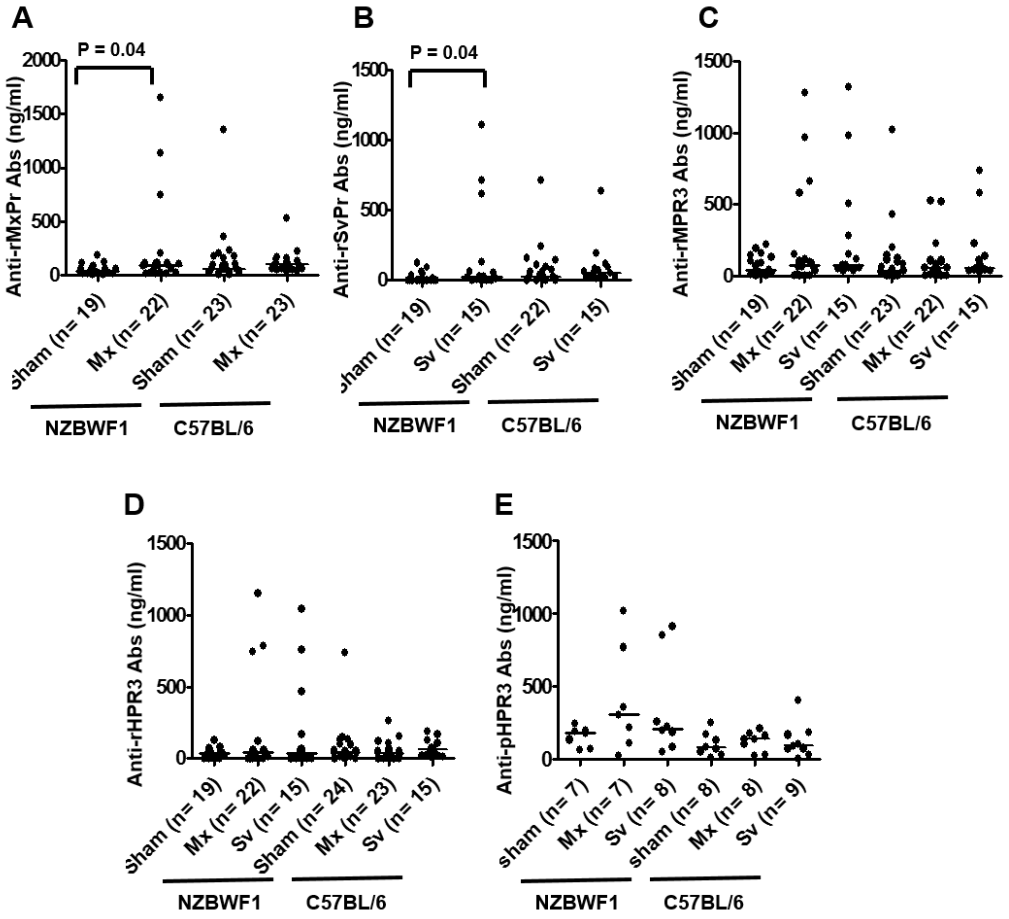
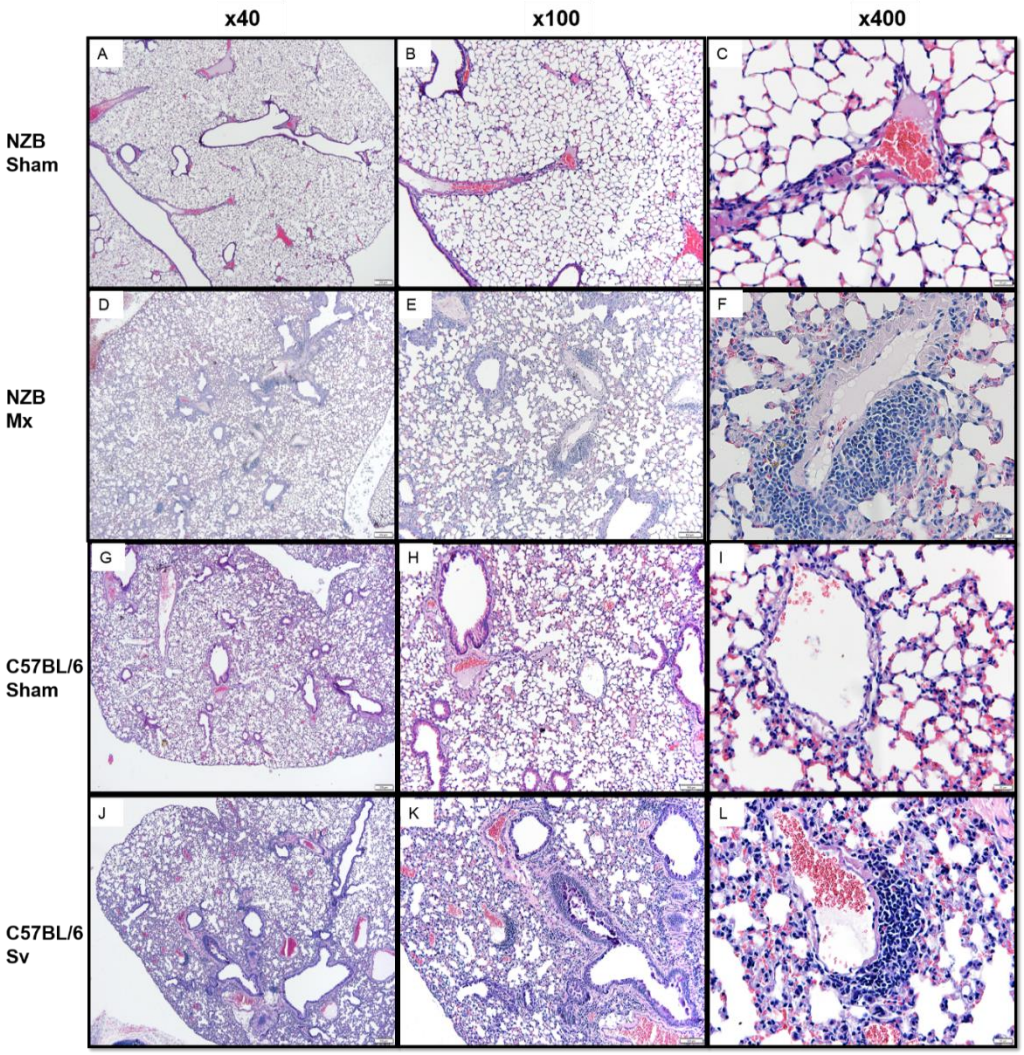


Figure 5. Nasal infection with Mx or Sv induced autoantibodies only in a few mice.

The NZBWF1 and C57BL/6 mice were intranasally infected by inoculation of 30 μl of 10^8 cells of Mx or Sv live bacteria in PBS onto the nostrils at 0, 1, 3 and 5 weeks. The sham group was received quantities of PBS. All mice were euthanized 10 days after the last infection. The levels of antibodies to, rMxPr (A), rSvPr (B), rmPr3 (C) rhPr3 (D), and phPR3 (E) in sera were measured by ELISA.

3.2.4. Nasal infection of Mx or Sv did not induce vasculitis or granuloma in lung or glomerulonephritis in kidney

Mice infected with PBS (Fig. 6 A, B, C), Mx (Fig. 6 D, E, F), or Sv (Fig. 6 J, K, L) developed inflammation in the lung, which presented as perivascular inflammatory infiltrations, but such inflammations were not observed in the sham controls (Fig. 6 M). However, neither vasculitis nor granuloma were detected in mice, even in mice that produced high levels of antibodies to rmPr3. Kidney tissues presented as normal in all PBS-, Mx-, or Sv-infected mice.


x40
x100
x400
NZB
Sham
NZB
Mx
C57BL/6
Sham
C57BL/6
Sv
A
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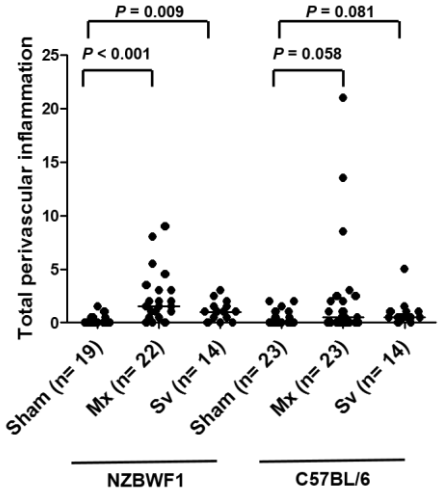


Figure 6. Although nasal infection of Mx or Sv alone increased perivascular inflammation in the lung, it did not induce either vasculitis or granuloma in lung. Light micrographs illustrating the histopathological examination of lung in the mice infected with Mx or Sv**.** The NZBWF1 and C57BL/6 mice were intranasally infected by inoculation of 30 µl of10^8 cells of Mx or Sv live bacteria in PBS onto the nostrils at 0, 1, 3 and 5 weeks. The sham group was received quantities of PBS. All mice were euthanized 10 days after the last infection. (A), (B), (C): the lung of sham NZBWF1 mouse infected with PBS, H&E. (D), (E), (F): the lung of NZBWF1 mouse infected with Mx, H&E. (G),(H),(I) : the lung of sham C57BL/6, H&E. (J),(K),(L): the lung of C57BL/6 mouse infected with Sv, H&E. The number of peri-vascular inflammation foci was counted (M).

3.3. Mice co-infected with Sa and Mx or Sv produce antibodies to bacteria

Whether infected with Mx or Sv, only a few mice produced ANCA. Moreover, organ pathologies were not detected in Sv- or Mx-infected mice. Polymicrobial infections that include Sa produce enhanced disease severity and morbidity in GPA. Therefore, co-infection of Sa with Mx or Sv was undertaken to determine if such co-infection could enhance ANCA production or granuloma formation. During the course of their infection with Mx or Sv, mice were infected once with Sa. The Sa co-infection did not affect the antibody responses to the Mx or Sv lysates. All mice co-infected with Sa and Mx produced antibodies to Mx lysate in both the NZBWF1 and C57BL/6 mouse strains (Fig. 7 A), whereas, all mice co-infected with Sa and Sv produced antibodies to the Sv lysates in both mouse strains (Fig. 7 B), as was observed in mice infected with Mx or Sv alone.

Mice did not produce antibodies to rMxPr even though, in mice co-infected with Sa and Mx, autoantibodies to rmPr3 were detected in 18% of NZBWF1 and 20% of C57BL/6 mice (Fig. 7 E). In contrast, co-infection of Sa with Sv produced autoantibodies to rmPr3 in 16% of NZBWF1 and 0% of C57BL/6 mice. When compared with the sham (Sa infected) controls, only the co-infected NZBWF1 mice showed statistically significant higher levels of antibodies to rmPr3 ($P < 0.05$). Therefore, co-infection of Sa did not enhance the production of antibodies in response to the tested proteases. Perivascular inflammation was detected more in the NZBWF1 mice than in the sham controls (Fig. 8 M). Interestingly, one C57BL/6 mouse co-infected with Sv and Sa developed a necrotic granulomatous lesion in the lung in the absence of a high level of autoantibodies to rmPr3 (Fig. 8 D, E, F).

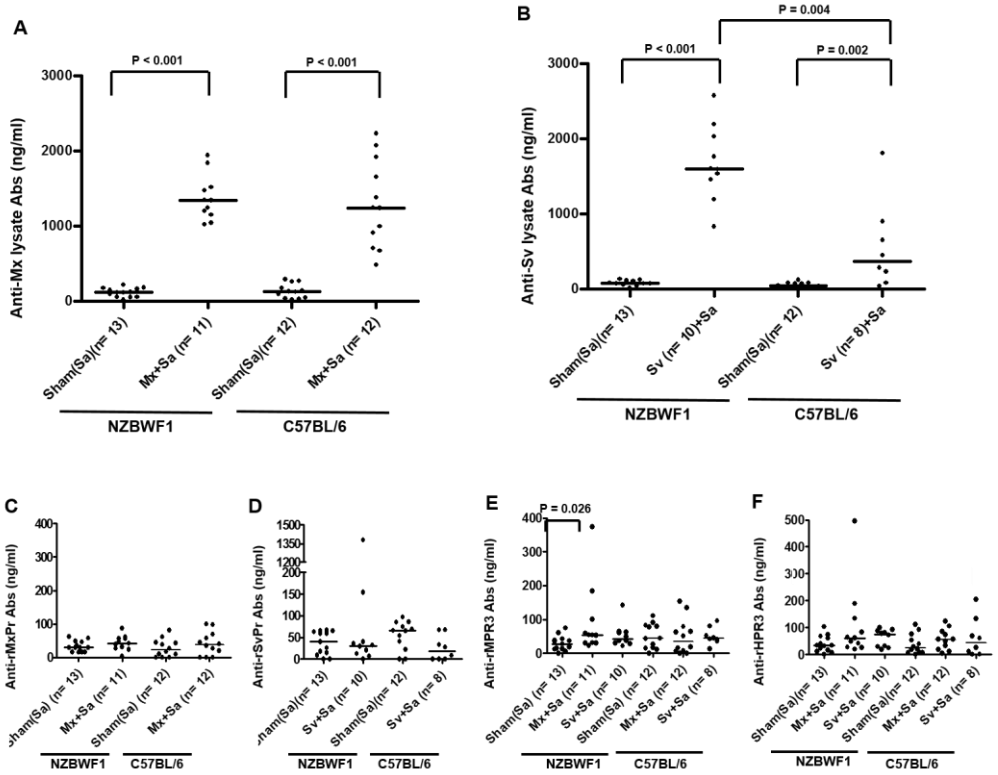
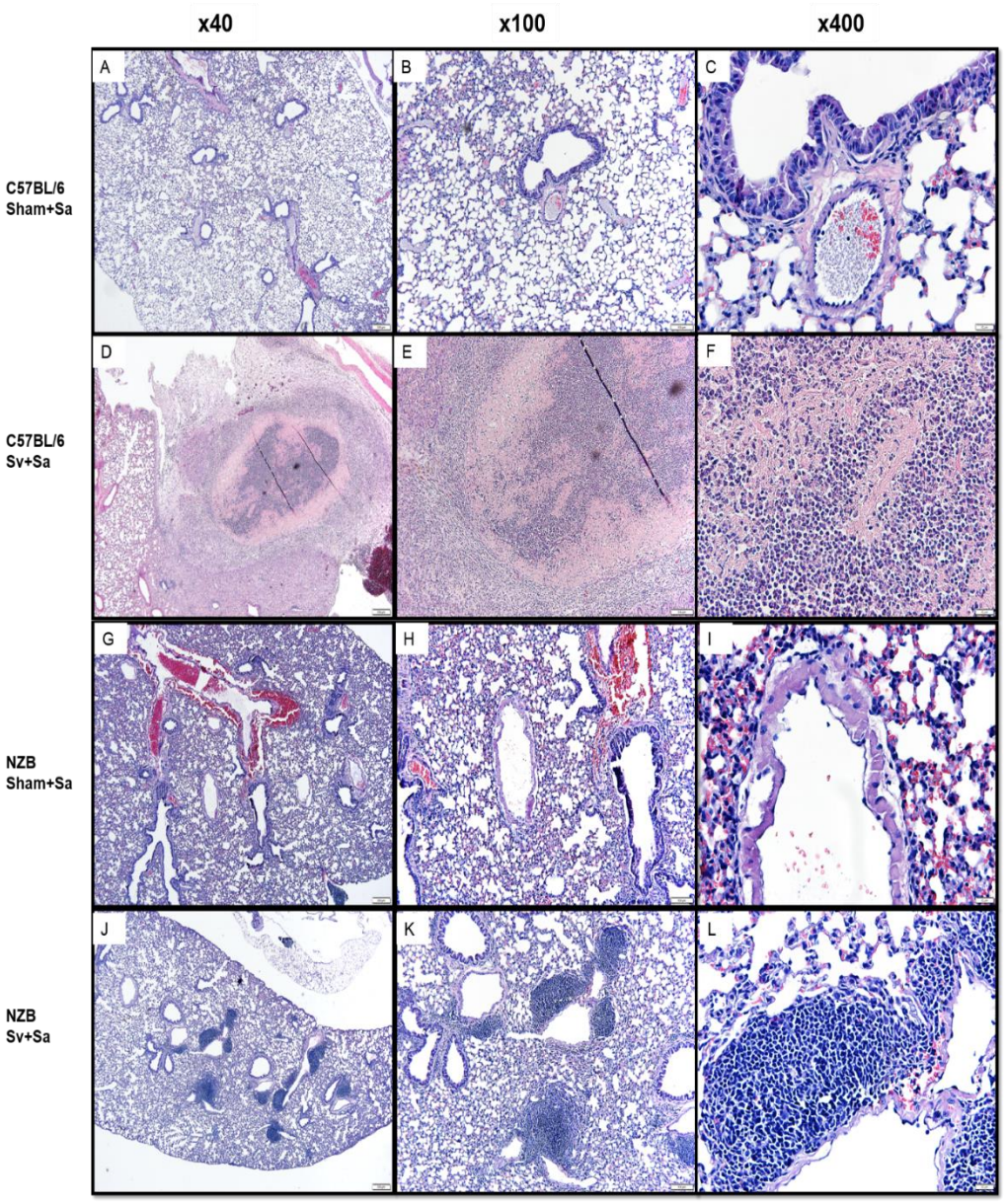


Figure 7. Although Mice co-infection of *Sa* with Mx or Sv produce antibodies to bacteria, induced autoantibodies only in a few mice.

The NZBWF1 and C57BL/6 mice were intranasally infected by inoculation of 30 µl of10^8 cells of Mx or Sv live bacteria in PBS onto the nostrils at 0, 1, 3 and 5 weeks. The mice were co-infected with 10^6 cells of Sa together with Mx or Sv on time at either 1 or 5 weeks. The sham (Sa) group was received PBS or Sa alone. The level Antibodies to Mx lysates (A), Sv lysate (B), rMxPr (C), rSvPr (D), rmPr3 (E), and rhPr3 (F) in sera of mice infected with Mx or Sv with Sa


x40
x100
x400
A
B
C
C57BL/6
Sham+Sa
D
E
F
C57BL/6
Sv+Sa
G
H
I
NZB
Sham+Sa
J
K
L
NZB
Sv+Sa

M.

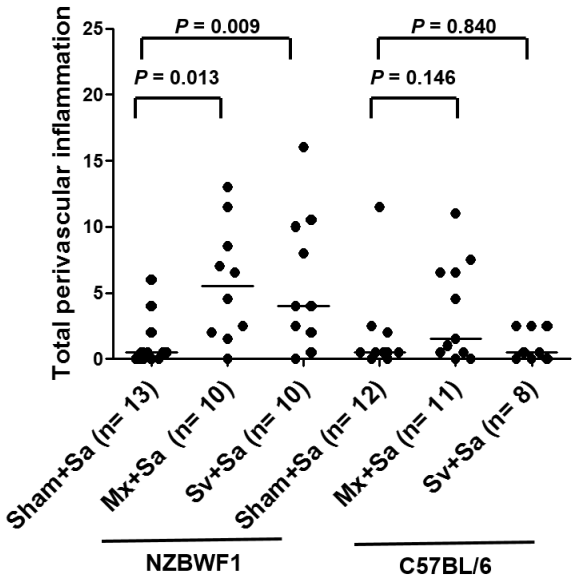


Figure 8. NZBWF1 mice develop PR3-ANCA and peri-vascular inflammation. Interestingly, only one mouse developed granulomatous inflammation. The NZBWF1 and C57BL/6 mice were intranasally infected by inoculation of 30 µl of10^8 cells of Mx or Sv live bacteria in PBS onto the nostrils at 0, 1, 3 and 5 weeks. The mice were co-infected with 10^6 cells of Sa together with Mx or Sv on time at either 1 or 5 weeks. The sham (Sa) group was received PBS or Sa alone. Light micrographs illustrating the histopathological examination. (A), (B), (C) the lung of sham+Sa C57BL/6 mouse. (D), (E), (F): the lung of C57BL/6 mouse co-infected of Sa with Sv, H&E. (G), (H), (I): the lung of sham+Sa NZBWF1 mouse, H&E. (J), (K), (L): the lung of C57BL/6 mouse co-infected of Sa with Sv, H&E. The number of peri-vascular inflammation foci was counted (M).

3.4. Mice immunized with rSvPr produce antibodies to rmPr3, rSvPr, rhPr3, and phPR3 (PR3-ANCA) and exhibit lung inflammation

To determine whether immunization with rSvPr can induce the formation of antibodies to SvPr and mPR3, mice were immunized with rSvPr. Antibody responses to rmPr3 (Fig. 9 A-1) and rSvPr (Fig. 9 A-2) were detected two weeks after the first immunization and the response increased after the second boosting. Therefore, antibody levels were assessed three weeks after the second boosting. In contrast to the results of bacterial infection, immunization with rSvPr resulted in production of antibodies to rSvPr in 91% of NZBWF1 and 100% of C57BL/6 mice. In addition, antibodies to rmPr3 were detected in 75% of NZBWF1 and 100% of C57BL/6 mice (Fig. 9 C). Therefore, almost all mice immunized with rSvPr produced autoantibodies. In addition, most of the rmPr3-reactive sera reacted to rhPr3 and phPR3. Interestingly, C57BL/6 mice exhibited a greater immune response than that in NZBWF1 mice.

In contrast to sham control mice (Fig. 10 A, B, C), the mice immunized with rSvPr developed vasculitis. Moreover, destruction of blood vessels and presence of various types of immune cells were detected in lung tissue (Fig. 10 F, L). Although typical forms of vasculitis were not detected, small- to medium-sized blood vessel filled with inflammatory cells were observed. There was no overt abnormal findings in kidney. Interestingly, C57BL/6 mice developed more parenchymal inflammation loci than those in NZBWF1 mice (Fig. 10 M).

To determine whether antibodies to mouse PR3 were present in the sera of mice immunized with rSvPr, a Western blot analysis was performed using serum from a NZBWF1 mouse immunized with rSvPr to mouse neutrophil, rmPR3, rhPr3, rSvPr, and *E. coli* lysates. In that serum, the 25 kDa mouse Pr3 protein band was present (Fig. 10

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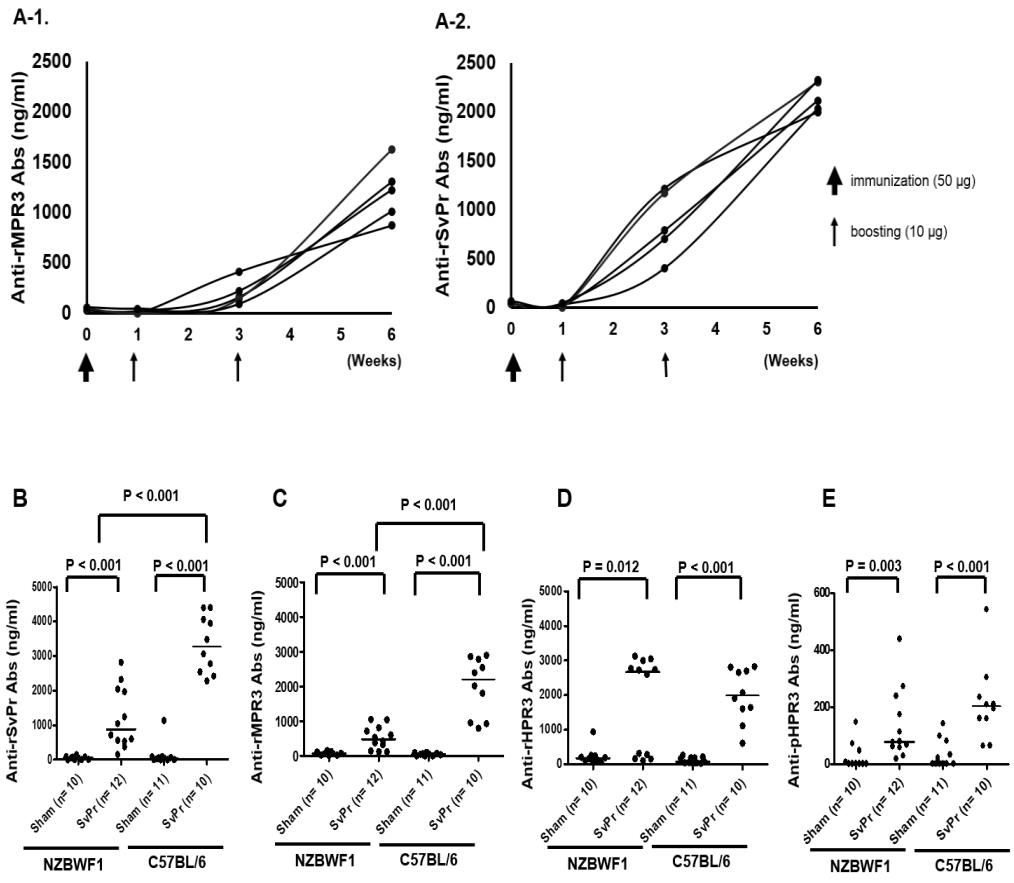
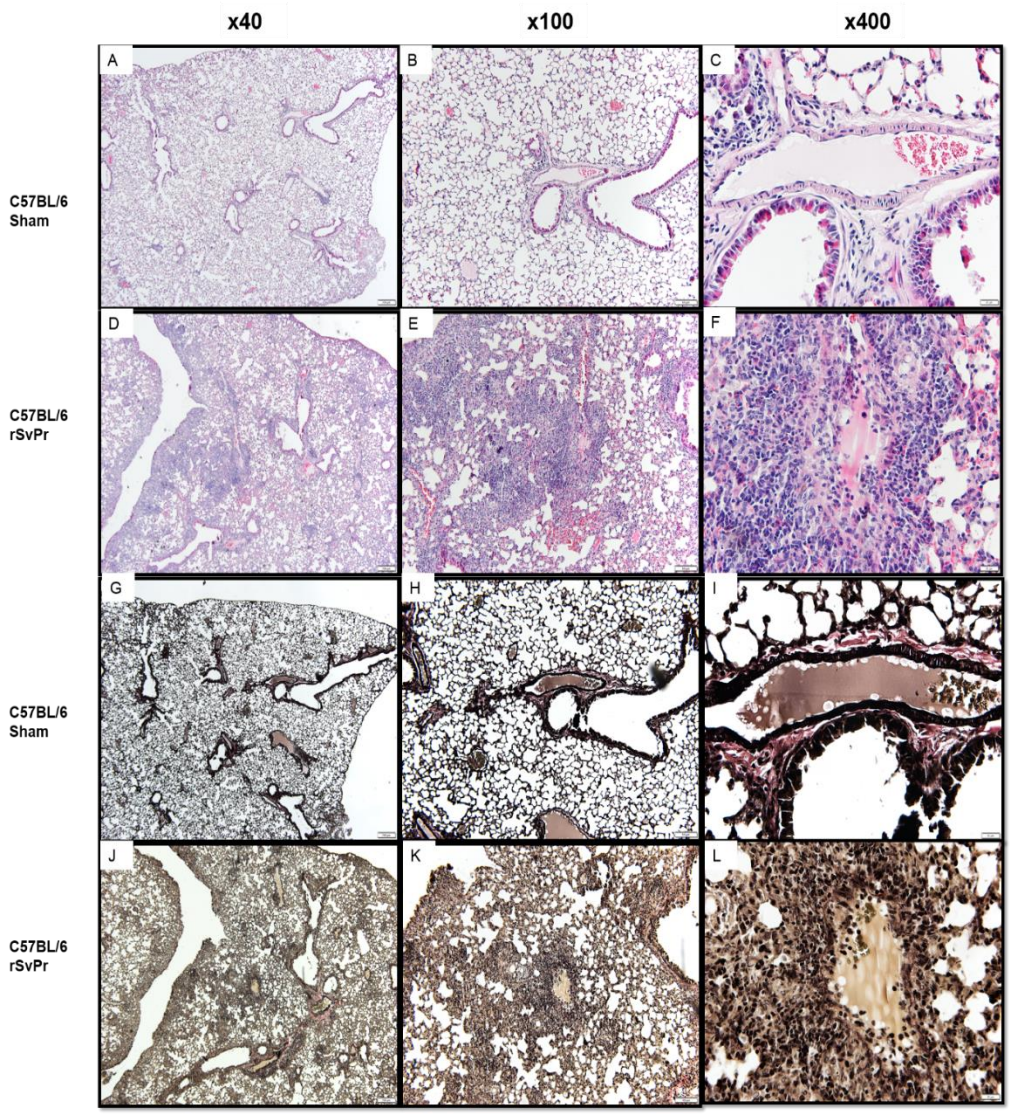


Figure 9. Immunization of rSvPr induced antibodies to rmPr3, rSvPr, rmPr3, rhPr3, and phPR3. Three- to four- week old mice were immunized subcutaneously with 50 µg of *Sv* protease emulsified with CFA. One and three weeks later mice were boosted again with the 10 µg of Sv protease emulsified with CFA. The sham group was immunized equal quantities of vehicle emulsified with complete Freund's adjuvant. The immunized mice were euthanized 2 weeks after the last immunization. Antibodies to rmPr3 (A-1) and rSvPr (A-2) in sera of mice immunized with rSvPr at various time points in the

NZBWF1 mouse. Antibodies to rSvPr (B), rmPr3 (C), rhPr3 (D), and phPR3 (E) were determined ELISA in sera of mice immunized with rSvPr.


x40
x100
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A
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C57BL/6
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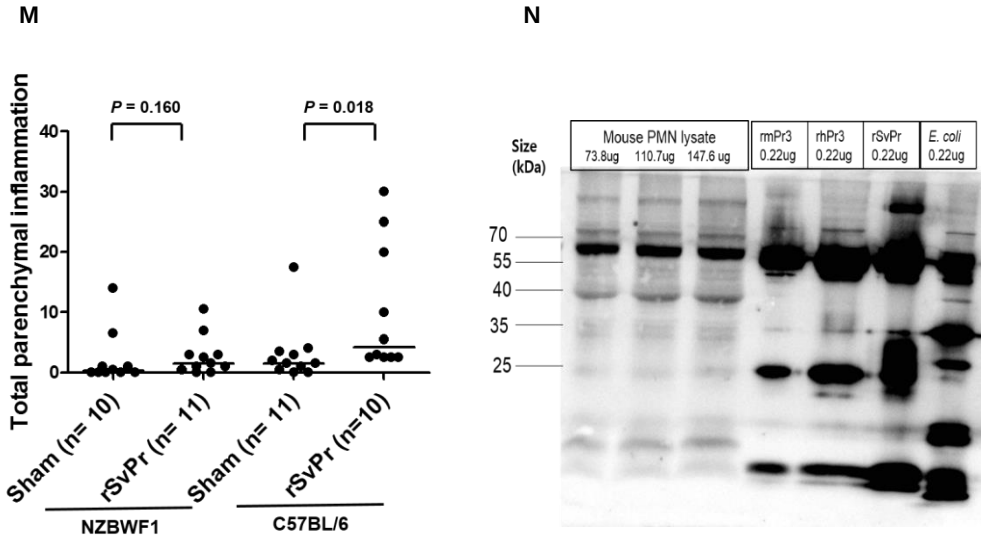

Figure 10. Mice immunized with rSvPr induce inflammation and vasculitis in the lung. Three- to four- week old mice were immunized subcutaneously with 50 µg of rSvPr emulsified with CFA. One and three weeks later mice were boosted again with the 10 µg of rSvPr emulsified with CFA. The sham group was immunized equal quantities of vehicle emulsified with complete Freund's adjuvant. The immunized mice were euthanized 2 weeks after the last immunization. Light micrographs illustrating the histopathological examination. (A), (B), (C) the lung of C57BL/6 mouse immunized CFA, H&E. (D), (E), (F): the lung of C57BL/6 mouse immunized with rSvPr, H&E. (G), (H), (I) the lung of C57BL/6 mouse immunized CFA, Verhoff's strain. (J), (K), (L): the lung of C57BL/6 mouse immunized with rSvPr, Verhoff's strain. The number of parenchymal inflammation was counted (M). Western blot was performed using serum from a NZBWF1 mouse immunized with rSvPr for the detection of PR3 of mouse neutrophils.

3.5. Additional infection produces perivascular inflammation and vasculitis in the ANCA-induced mice

Because ANCA inhibits the clearance of bacteria by neutrophils, it was hypothesized that a bacterial infection in the presence of ANCA may develop granuloma. In addition, inflammatory cytokines induced by bacterial infection or LPS may aggravate ANCA-mediated vasculitis or granuloma. To test these hypotheses, mice were immunized with rSvPr to induce ANCA production and were then challenged once with Sa (nasal), Sv (nasal), or LPS (intraperitoneal). After the challenge, inflammatory infiltration in the perivascular areas (Fig. 11 A), granulomas (Fig. 11 B, C-1), and vasculitis (Fig. 11 C-2) were detected in lung tissue.

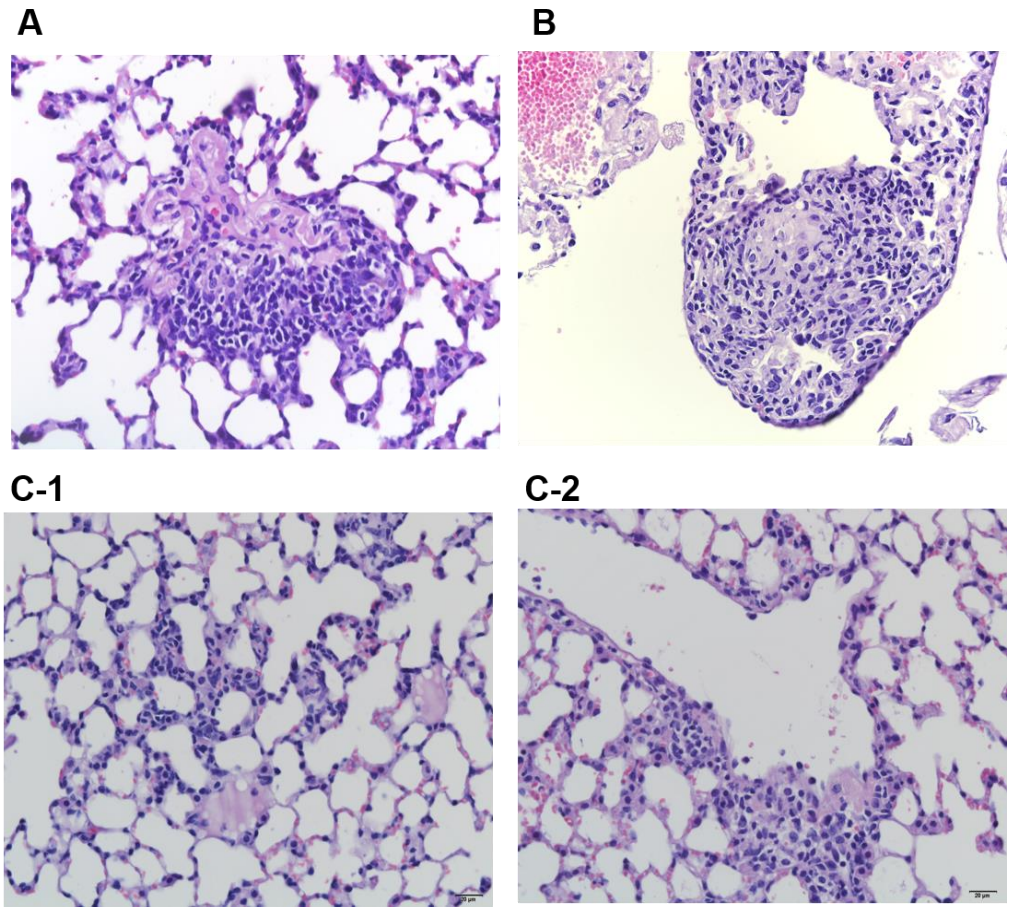

Figure 11. Additional infection induced vasculitis and granulomatous inflammation in the ANCA-induced mice. Light micrographs illustrating the histopathological examination of lung from the mice challenged with Lipopolysaccharides (LPS), Sa, or Sv after ANCA-induction by immunization with rSvPr. The lung of C57BL/6 mouse challenge with LPS after ANCA-induction by immunization with rSvPr (A). The lung of C57BL/6 mouse 20 days after the challenge with Sa after ANCA-induction by immunization with rSvPr (B). The lung of C57BL/6 mouse after 20 days the challenge with Sv after ANCA-induction by immunization with rSvPr (C). H&E; (A), (B), and (C). Original magnification; (A), (B), and (C) x 400

3.6. Mice subcutaneously infected with Sv produce antibodies to rmPr3, rSvPr, rhPr3, and phPR3 at low levels

To elucidate the molecular mechanism involved in the efficient production of autoantibodies following rSvPr immunization, C57BL/6 mice were infected subcutaneously with Sv emulsified with CFA. Such immunization induced a high level of antibody response to the Sv lysate (Fig. 12 A); however, there were low levels of antibody response to rSvPr (Fig. 12 B), rmPr3 (Fig. 12 C), rhPr3 (Fig. 12 D), and phPR3 (Fig. 12 E).

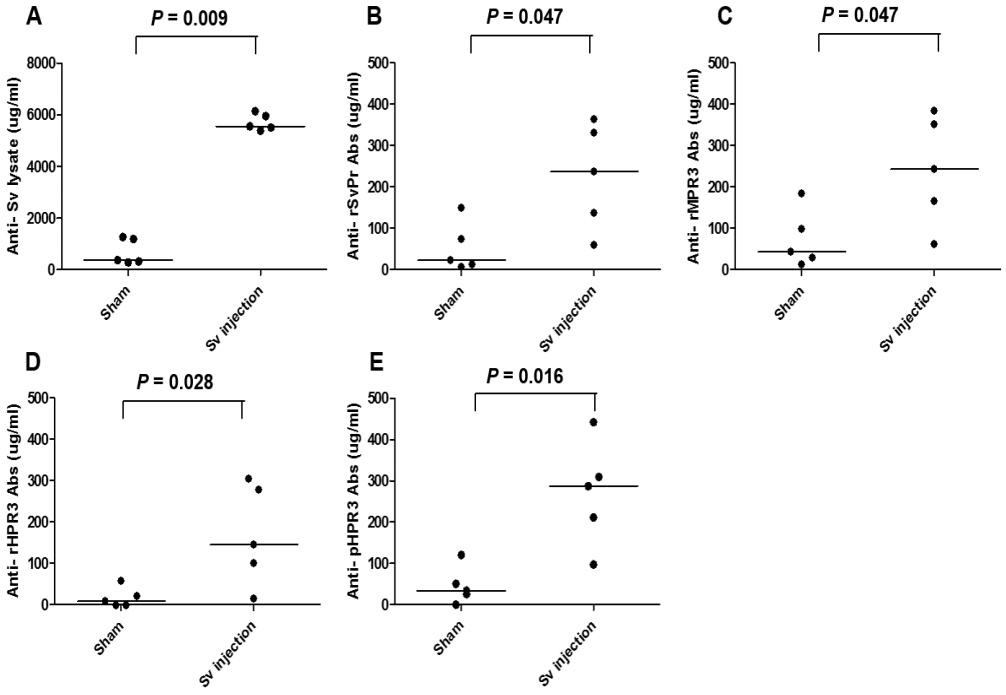

Figure 12. Mice immunized with Sv produced antibodies to rmPr3, rSvPr, rhPr3, and phPR3 at low levels. Three- to four- week old mice were immunized subcutaneously with 10^8 cells of *Sv* emulsified with CFA. One and three weeks later mice in each group were boosted again with the 10^8 cells of the same materials. Antibodies to Sv lysate (A), rSvPr (B), rmPr3 (C), rhPr3 (D), and phPR3 (E) in sera of mice immunized with Sv in C57BL/6 male mice.

3.7. GPA patients had antibodies to Sv lysate and rSvPr

Antibody responses to bacteria, bacterial proteases, antibodies to phPR3, Sv lysates, rSvPr, rMxPr, rhPr3, Mx lysate, and Sa lysate were measured in GPA patient sera. Among the sera samples from 19 GPA patients, 7 samples showed positive reactivity to purified human PR3 (Fig. 13 A), whereas 6 samples reacted positively to Sv lysate (Fig. 13 B). Among those 6 samples, three samples also reacted positively to rSvPr (Fig. 13 C), rMxPr (Fig. 13 D), and rhPr3 (Fig. 13 E). In contrast, 12 of the 19 normal samples reacted to Sv lysates, but only one of the 19 reacted to rSvPr and that reaction was at a very low level.

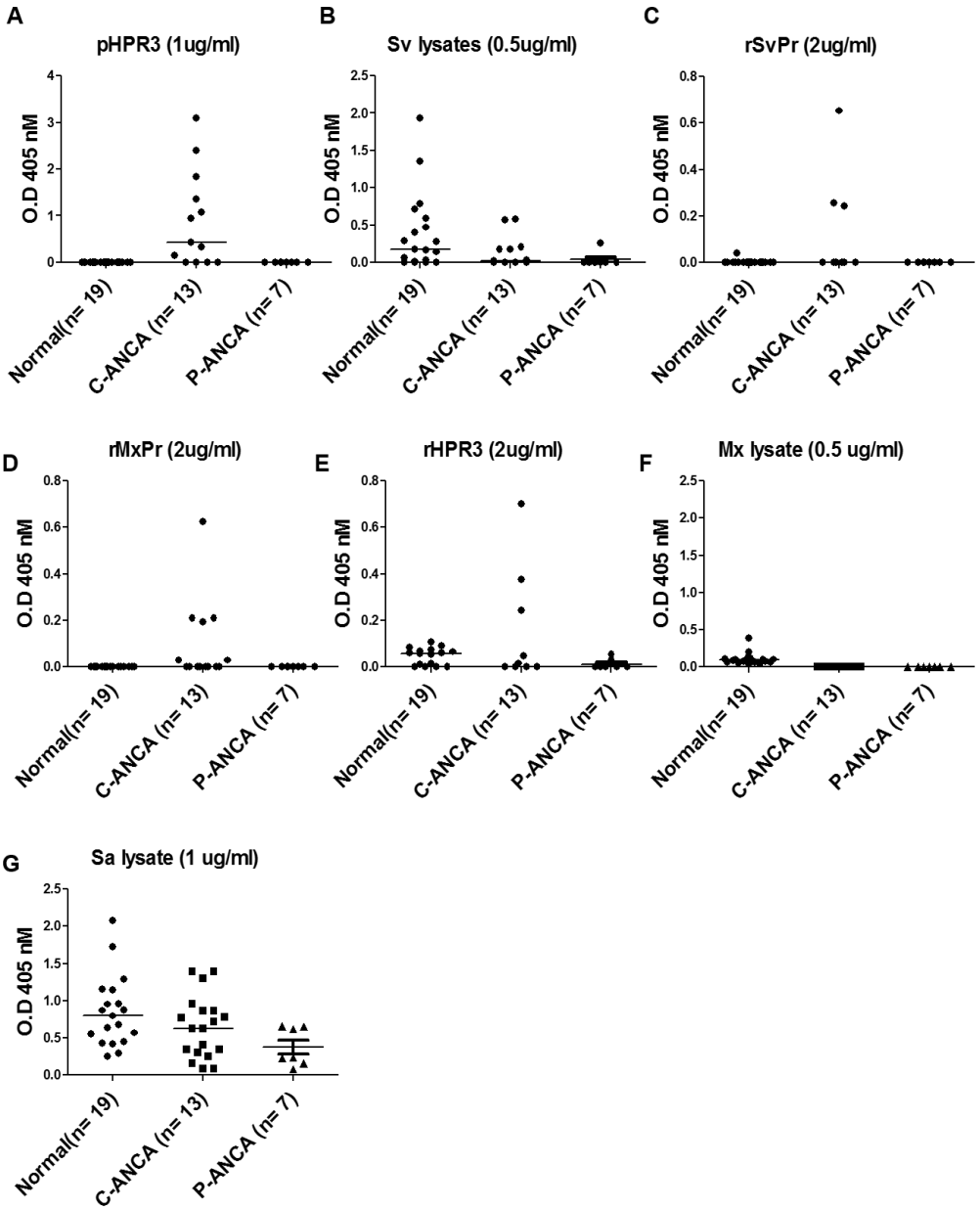

Figure 13. GPA patients had antibodies to Sv lysate and rSvPr. Antibodies to phPR3 (A), Sv lysate (B), rSvPr (C), rMxPr (D), rhPr3 (E), Mx lysate (F) and .Sa lysate (G) in sera of GPA(C-ANCA: 13 patients, P-ANCA: 7 patients) patients and control subjects.

4. Discussion

The present study provides evidence that ANCA production can be induced via molecular mimicry following infection by bacteria that possess PR3 homologous proteins.

In response to nasal infection by either Mx or Sv, NZBWF1 mice produced a higher level of autoantibodies to rmPr3 than that produced in C57BL/6 mice. In addition, the NZBWF1 mice were more induced autoantibodies to rmPr3 than C56BL/6 mice. It was expected that NZBWF1 mice could produce autoantibodies because that strain is autoimmune prone. However, the rSvPr immunization results showed that C57BL/6 mouse produced antibodies to rmPr3; thus, there was no need to include an autoimmune prone mouse strain in this study. Perivascular inflammation was induced in lung tissue in NZBWF1 mice to a greater extent than that in C57BL/6 mice. when compared to those sham control. Those results indicate that NZBWF1 mice are more sensitive than C57BL/6 mice to bacterial infection. One possible explanation of that result is that, if NZBWF1 mice have a dysfunctional epithelial barrier, bacteria could infect the mouse efficiently resulting in antibody production to the infecting bacteria and its components. However, there were no statistically significant differences in antibody production between mouse strains or bacteria types. In mice co-infected with Sa and Mx or Sv, there were differences in the production of antibodies to the different protease treatments. However, when compared with single bacterial infection with Mx or Sv, there were no statistically significant differences. These results suggest that the production of autoantibodies to autoantigens can be eliminated during the production of antibodies against bacterial agents that possess proteins that are homologous to those in the host. However, some mice with a disorder that affects the elimination of autoantibodies can develop antibodies to autoantigens.

A remarkable finding was that almost all mice developed autoantibodies to rmPr3 following immunization with rSvPr. Production of autoantibodies to rmPr3 was observed in 75% of NZBWF1 mice and 100% of C57BL/6 mice. In both strains, the number of autoantibody producing mice and the level of autoantibody production were increased by rSvPr immunization when compared to the results following infection of Mx or Sv only. Moreover, C57BL/6 mice produced a higher level of autoantibody production to rmPr3 than that in NZBWF1 mice. This result may be attributed to the larger body size of NZBWF1 mouse than that of C57BL/6 mouse. Regardless, it is possible that, following immunization with an autoantigen homologous protein, even a non-autoimmune mouse strain can produce the autoantibody. Similarly, immunization with bacteria induced the production of autoantibodies to rmPr3 in four of five of the tested C57BL/6 mice. There are two possible explanations for these results. First, if Sv-immunized mice produce less PR3 homologous protease than rSvPr-immunized mice that may produce the difference in the levels of production of autoantibodies in the two groups. Second, only five mice were used in the assessment of immunization and autoantibody production, an inadequate sample size for rigorous statistical testing. Therefore, further study is required to elucidate the differences between the effects of immunization of rSvPr and Sv in mice.

It has been reported that deregulation of BAFF, an important regulator of the B cells proliferation, is associated with autoimmune diseases such as Sjögren's syndrome [85]. Moreover, elevated BAFF expression has been detected in mice intranasally infected with *Pseudomonas aeruginosa* [86]. It means that mice can be produced.

Although, bacterial infection produced ANCA in mice granulomas were not detected, thus suggesting that ANCA production does not lead to granuloma formation.

However, the ANCA-positive mice were not assessed over a long follow-up period in this study. Interestingly, only one C57BL/6 mouse co-infected with Sv and Sa had a necrotic granulomatous lesion. It is possible that additional effects related to co-infection will induce granulomatous lesions. In addition, small granulomatous lesions were detected in mice that were challenged with LPS, Sa, or Sv following induction of antibodies to rmPr3 by immunization with rSvPr. Thus, it is possible that additional bacterial infection in ANCA-induced mice can lead to the formation of granulomatous inflammation. Inhibition of phagocytosis of bacteria, (i.e., clearance of infection), by ANCA may result in collections of immune cells, such as macrophages and lymphocytes, and lead to the formation of granulomatous inflammation.

Vasculitis was detected in the lung of mice immunized with rSvPr indicating that mice can produce ANCA via immunization by a PR3 homologous protein. Only ANCA can activate mouse neutrophils, which may then enter blood vessels within the lung. Moreover, additional challenges following immunization with rSvPr also induced vasculitis in the ANCA-induced mice, thus indicating that additional infection can aggravate vasculitis.

The bacterial protein database used in this study is updated frequently and is publicly available. To obtain more recent search results on bacteria with a PR3 homologous protease, the database was searched a final time on 23 May 2014. Interestingly, since the original search date used in this study, many bacteria with higher homologies than those of Mx and Sv were now in the database. Although not previously reported in human infection, the newly listed *E. coli* 1140 had the highest homology (35%/53%, identities/positives) with PR3. Another newly listed bacteria, *Enterobacter cloacae*, had 31%/48% identities/positives with hPR3. *E. cloacae*, a nosocomial

pathogen, can act as an opportunistic pathogen infecting skin as well as the gastrointestinal and urinary tracts [87]. Based on the results in this study of Mx and Sv, those two bacteria may also be capable of inducing the production of autoantibodies to PR3 via molecular mimicry.

Collectively, the results suggest that infection by bacteria containing PR3-homologous proteases can induce PR3-ANCA production via molecular mimicry and may result in lung symptoms such as vasculitis.

5. Conclusion

In mice, infection with bacteria containing PR3-homologous proteases or immunization with bacterial PR3-homologous proteases can induce PR3-ANCA production via molecular mimicry. Although elevated ANCA production did not induce granuloma development, ANCA alone did produce lung inflammation, which presented as inflammatory cell infiltration in the perivascular areas and as vasculitis involving small- to medium-sized blood vessels.

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국문초록

다발혈관염 육아종증 마커인 C-ANCA 형성에 관여하는 PR3 상동세균 단백 분해 효소의 역할

김 용 철

서울대학교 치의학대학원

구강악안면 감염 및 면역학 전공

(지도교수 : 최 영 님)

목적

발병 원인이 잘 알려지지 않은 자가면역질환인 다발혈관염 육아종증 (Granulomatosis with polyangiitis, GPA) 이 사람의 자가항원인 Proteinase 3 와 높은 상동성을 발현하며 인체에 침입 할 수 있는 세균에 대한 면역 반응의 결과로 발생

한다는 가설을 전제로 베게너 육아종에 대한 동물 모델의 확립, 세균과 세균의 protease 에 대한 항체와 베게너 육아종의 상관관계 연구, 자가면역질환 환자의 시료 연구를 통하여 검증하고자 한다.

실험 방법

본 연구를 위해서 사람 PR3 (rHPR3), 생쥐 PR3 (rmPR3), *S. viridis* protease (rSvPr) 그리고 *M. xanthus* protease (rMxPr) 단백질을 유전자 재조합 기술을 이용하여서 *Escherichia coli*에서 발현 시켜 추출 하였다. 생쥐는 자가면역질환 모델인 NZBWF1 과 정상 생쥐인 C57BL/6를 사용 하였다. 세균 자체의 효과를 보기 위하여 생쥐를 *M. xathus* 와 *S. viridis*를 각각 감염 시켰다. 또한, 복합 감염의 효과를 보기 위하여 다발혈관염과 관련이 있다고 보고되고 있는 *Staphylocossu aureus*를 이용하여 생쥐를 복합 감염을 시켰다. 세균 단백질에 대한 반응을 보기 위하여 PR3 와 상동성이 있는 재조합 *S. viridis* protease 의 단백질을 이용하여 생쥐를 감염 시켰다. 생쥐의 폐에서 육아종과 혈관염을 관찰하기 위하여 헤마톡실린과 에오신 염색을 수행 하였으며, 생쥐의 혈청의 항체를 분석하기 위해서 재조합 단백질 rHPR3, rmPR3, rSvPr, rMxPr, *M. xanthus* lysates 그리고 *S. viridis* lysates 에

대하여 효소결합면엽흡착검사 (Enzyme-linked immunosorbent assay, ELISA)를 수행하였다. 또한, 자가면역질환 환자(다발혈관염)의 혈청을 이용하여 세균, 세균의 단백질에 대한 항체 반응을 확인 하였다.

연구결과

PR3 상동 단백질을 가진 세균(*M. xanthus*, *S. viridis*)의 단독 감염 결과 세균 자체에 대한 항체는 모든 생쥐에서 생성 되었지만 생쥐의 자가 항체인 생쥐 PR3 에 대한 항체는 생쥐의 종과 감염 세균에 따라 9~20% 의 생쥐에서 생성 되었다. 조직학적 관찰에서는 생쥐의 폐에서 많은 염증세포의 침입을 볼 수 있었다. 이에 항체 생성의 증가와 육아종 및 혈관염을 관찰하기 위해서 GPA 환자들에서 만성적으로 감염이 나타나는 *Staphylocossu aureus* 을 공동감염 시켰다. *M. xanthus* 와 *S. viridis* 의 단독 감염에 비해 자가항체 및 PR3에 대한 항체는 증가는 없었으나 한 마리 생쥐의 폐에서 육아종증성 염증을 관찰 할 수 있었다. 이에 항체의 생성을 증가시키기 위해서 재조합 단백질인 recombinant *S. viridis* protease를 생쥐에 감염 시켰다. 그 결과 75% 의 NZBWF1 생쥐, 100% C57BL/6 생쥐에서 생쥐의 PR3 에 대한 자가 항체를 형성하고 적은 수의 생쥐 폐에서 혈관염을 관찰 할 수 있었다.

자가면역질환 환자(다발혈관염)의 혈청에서 19명 중 6명에게서 세균 *S. viridis*에 대한 항체가 관찰 되었으며, 그 중 3명에서는 rSvPr, rMxPr, rhPR3 에 대한 항체가 관찰 되었다.

결론

생쥐 모델에서 PR3 상동 단백질을 가진 세균, 세균의 PR3 상동 단백질의 감염을 통하여 생쥐의 PR3 에 대한 자가 항체가 분자 모방설을 이용하여 유도 되었다. 비록 증가된 생쥐의 PR3 에 대한 자가 항체가 육아종을 생성 시키지는 못하였지만, 증가된 생쥐의 PR3에 대한 자가 항체는 생쥐의 폐의 소(small), 중(medium)-혈관 주위에 많은 염증과 혈관염(vasculitis)를 야기했다.

주요어 : 다발혈관염 육아종증 (Granulomatosis with polyangiitis, GPA), 항호중구세포질항체,
Proteinase 3, 분자모방설

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